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In our original proposal we presented preliminary evidence suggesting a critical region for the modulation of BRCA1's transactivation activity and proposed the existence of an intramolecular interaction domain that masks the activation domain of BRCA1, thus impairing the recruitment of the machinery involved in transcription initiation. Our hypothesis was that BRCA1 transcriptional function is regulated by an auto inhibitory intramolecular interaction between regions encoded by exon 12 (and possibly part of exon 13) and the C-terminal region comprising the BRCT domains. We suggested that this mechanism might form the basis of the regulation of transcriptional activation by BRCA1. Our expectation was that a region that interacts and masks the activation domain would be identified in detail and would reveal one of the means by which BRCA1 activity is regulated. During the past four years the experiments performed seem to refute our original hypothesis. At this point, the experiments conducted here have refuted our initial hypothesis of a regulatory role for an intramolecular interaction in BRCA1. Nevertheless, the experiments revealed that a putative coiled-coil domain spanning exons 12 and 13 is important for transcription regulation.

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INTRODUCTION

During the past five years the main focus of the laboratory has been to elucidate the role of BRCA1 in tumor suppression and to identify cancer-associated alleles of BRCA1. Inherited mutations in *BRCA1* account for the majority of breast and ovarian cancer families and the gene has been found to be epigenetically inactivated in high-grade sporadic breast tumors. The human *BRCA1* gene codes for a nuclear phosphoprotein that participates in the cellular DNA damage response. However, despite concentrated efforts by many laboratories, its exact biochemical functions have remained elusive. Our working hypothesis is that BRCA1-mediated transcription activation is critical for its tumor suppressive action.

In our original proposal we presented preliminary evidence suggesting a critical region for the modulation of BRCA1's transactivation activity and proposed the existence of an intramolecular interaction domain that masks the activation domain of BRCA1, thus impairing the recruitment of the machinery involved in transcription initiation. Our hypothesis was that BRCA1 transcriptional function is regulated by an auto inhibitory intramolecular interaction between regions encoded by exon 12 (and possibly part of exon 13) and the C-terminal region comprising the BRCT domains. We suggested that this mechanism might form the basis of the regulation of transcriptional activation by BRCA1. Our expectation was that a region that interacts and masks the activation domain would be identified in detail and would reveal one of the means by which BRCA1 activity is regulated. During the past four years the experiments performed seem to refute our original hypothesis. Although it is difficult to rule out an intramolecular regulatory domain we conclude that it does not play a major role in regulating BRCA1-mediated transcriptional activity.

BODY

During these four years we successfully completed all tasks described in the original proposal and in the work statement (see below).

Task 1. **To map the sites involved in the intramolecular interaction in BRCA1 using the yeast two-hybrid assay.**

- We developed a series of plasmids (see Reportable Outcomes) for expressing the BRCA1 region aa 1366-1455 and smaller fragments as in-frame fusions with the GAL4 DNA binding domain (DBD) and activation domain (AD).
- We also developed a series of plasmids (see Reportable Outcomes) for expressing the BRCA1 minimal transactivation domain and smaller fragments as in-frame fusions with the GAL4 DBD and AD.
- We performed yeast two-hybrid assays using the interaction region and the MTD and confirmed that a region comprising exon 12 was indeed interacting with regions in the C-terminal domain of BRCA1. However, the high background found in many fragments of BRCA1 confounded the results. Since many constructs displayed this activity it became difficult to design experiments to avoid these confounding effects. We also found that protein expression levels varied significantly in the clones studied and generated high variability and low reproducibility of the results in assays performed in different days. We concluded that the mapping of the interaction site couldn't be done unequivocally using the yeast-based approach.
- We prepared a series of FLAG-tagged constructs (see Reportable Outcomes) to circumvent the problems described above.
- We expressed the tagged fragments in 293T human embryonic kidney cells and tested the interaction by immunoprecipitation and western blotting (Figure 1) using a wide variety of antibodies and conditions for lysis and immunoprecipitation (details can be found in the 2000, 2001 and 2002 reports). We could not detect any significant interaction between the different fragments. Recently we also performed experiments with the proposed HA and FLAG tagged vectors and also failed to detect any interaction. Taken together, these experiments suggest that the interaction may not be biologically significant.

Task 2. **To define the in vivo inhibition and the dominant negative activity of truncations of BRCA1.**

- We developed a series of plasmids (see Reportable Outcomes) for expressing the BRCA1 region aa 1366-1455 in mammalian cells.
- We extensively optimized transfections of breast cancer cell lines and in ovarian cancer cell lines (HCC1937, L56Br-C1, HeLa, SKOV-3, CAOV-2, MCF-7, NIH-OVCAR-3, 293T).
- We confirmed that expression of the interaction domain blocks the activity of BRCA1 in inducing a reporter luciferase gene but the effects was small and highly variable when using different fragments.
- We tested whether there was a difference in the *trans*-inhibition in breast cancer cell lines (HCC1937) versus ovarian cancer cell lines (SKOV-3 and CaOV-2). We could not detect any trans-inhibitory activity in those cell types and therefore there is no difference between breast and ovarian cancer cells. It is possible that very high levels of expression (such as those obtained in 293T cells) are needed to see an effect, which also raises a question about the biological significance of the intramolecular interaction.

Task 3. **To analyze which mutations abolish transcriptional activation.**

- We introduced mutations found in patients with hereditary breast and ovarian cancer (M1775R; A1708E; Y1853X) and also including those found in the putative interaction domain (H1402Y, L1407P and H1421H).
- We tested whether these mutations affect transcription activation of a reporter gene in yeast cells and confirmed that known cancer associated mutations were able to abolish transcription activation of a construct with very high activity. Most importantly, variant L1407P, which is predicted to cause disruption of the coiled-coil region, shows reduced activity. It is important to stress that is unusual for variants that are not in the BRCT domain to affect transcription by the carboxy-terminal of BRCA1.
- We developed yeast two-hybrid constructs containing the mutations and tested whether it modifies the strength of interaction (judged by activation of β-galactosidase production in yeast two-hybrid assays. In conclusion, although the experiments in Task 3 point to a role for sequences in exon 13 in the modulation of transcriptional activity by BRCA1 they do not support the model of a biologically relevant intramolecular interaction.

Task 4. **To make deletions of the inhibitory domain.**

- We developed plasmids for expressing BRCA1 with deletions of exons 12 and 13 and showed that neither deletion affected significantly transcription activation significantly when normalized to protein levels.
- Since we were not able to verify the interaction we could not identify the critical residues and therefore did not develop the constructs carrying missense mutations in this residue nor assayed their transcription activation capacity. Development of this task was modified because its aims depended on the identification and characterization of an interaction domain. Because the experiments in other tasks refuted our initial hypothesis this task was considered partially dispensable. Nevertheless, we generated constructs with deletions that will serve to further characterize the role of these sequences.

Task 5. **To map the phosphorylation sites involved in modulation.**

Several of the experiments described in Task 5, which involved the identification of putative phosphorylation sites, were superseded by the identification of phosphorylation sites of BRCA1.

- We introduced point mutations in putative phosphorylation sites S1423A, S1457A, S1497A, S1524A, S1542A.
- We were not able to confirm that the individual mutants fail to be phosphorylated probably because the construct always gets some level of phosphorylation in the other sites that have not been mutated.
- We developed GAL4 DBD fusion proteins of BRCA1 with disrupted phosphorylation sites. We have determined that these mutations do not affect transcription activation in (non-irradiated) cells and therefore

will be useful to analyze the role of phosphorylation since the artificial mutation *per se* does not affect transcription.

KEY RESEARCH ACCOMPLISHMENTS

- Development of a reproducible functional assay system to classify missense variants of BRCA1 that are located between aa 1366 and 1864 greatly expanding the scope of the assay. We are now in the process of expanding the assay to account for missense mutants in the full-length protein.
- Using our structure function analysis of BRCA1 we have developed of a structure-based algorithm to predict the outcome of all missense mutations in the BRCT region of BRCA1 (Mirkovic et al., submitted to publication).
- Determined that mutations in exon 13 that are predicted to disrupt the putative coiled-coil domain affect transcription activation revealing that this domain might be important for the tumor suppressive action of BRCA1. We are currently further characterizing this region.
- Determined that Serine to alanine mutations in the putative phosphorylation sites do not modify transcription activity in non-induced (non-irradiated) cells, thus allowing the use of these reagents to understand the role of phosphorylation in transcription activation.
- We performed a yeast two-hybrid screen to identify interacting partners to sequences in exon 12 and 13. This screen identified a cDNA coding for Talin as a strong binding partner to BRCA1. This interaction was also confirmed by a very stringent mammalian two-hybrid assay. We are now characterizing this interaction using complementary methods, such as immunoprecipitation.
- We identified and characterized a series of conditional BRCA1 mutants in transcription activation that display temperature-sensitiveness in transcription activation.
- Recently we have identified specific post-translational modification of histone H2AX that control its turnover in response to DNA damage.

SUMMARY OF TASKS

- Task 1: *All experiments were performed and Task 1was completed.*
- Task 2: *All experiments were performed and Task 2 was completed.*
- Task 3: *All experiments were performed and Task 3was completed.*
- Task 4: *All experiments were performed and Task 4 was completed.*
- Task 5: *All experiments were performed and Task 5 was completed.*

REPORTABLE OUTCOMES

Reagents (Plasmids and yeast strains)

- A yeast strain with an inducible expression of human BRCA1. TGYBRU. Yeast strain made on a TGY14 background expressing an integrated single copy wild type full length BRCA1 under the control of an inducible (GAL1) promoter.

- A series of yeast and mammalian expression plasmids.

GAL DBD and AD fusions:

pGBT9 BRCA1 (aa 1366-1803)	pGBT9 BRCA1 (aa 1396-1778)
pGBT9 BRCA1 (aa 1366-1778)	pGBT9 BRCA1 (aa 1366-1803)
pGBT9 BRCA1 (aa 1366-1718)	pGBT9 BRCA1 (aa 1366-1559)
pGBT9 BRCA1 (aa 1-302)	pGBT9 BRCA1 (aa 302-1313)
pGBT9 BRCA1 (aa 1560-1803)	pGBT9 BRCA1 (aa 1366-1455)
pGAD BRCA1 (aa 1366-1778)	pGAD BRCA1 (aa 1560-1863)
pGAD BRCA1 (aa 1-1863)	pACT2 BRCA1 (aa 1-1863)

No fusions:

p425GPD BRCA1 1853X	p425GPD BRCA1	p425GPD BRCA1 C61G
p425ADH BRCA1	p424GPD BRCA1	pJG4-4 BRCA1

Integrating vectors:

BB25 BRCA1	BB301 BRCA1
FLAG-tagged vectors:	
pCMV-FLAG (aa 1366-1778)	pCMV-FLAG (aa 1366-1455)
pCMV-FLAG (aa 1366-1559)	pCMV-FLAG (aa 1366-1718)
pCMV-FLAG (aa 1-302)	pCMV-FLAG (aa 302-1313)
GAL4 DBD fusions:	
pSG424 (aa 1396-1863)	pCDNA3 SG (aa 1560-1863)
pCDNA3 SG (aa 1396-1863)	
No fusions:	
pCDNA3 (aa 1366-1778)	pCDNA3 (aa 1560-1863)
pCDNA3 1853X	

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Funding obtained

NIH RO1 CA92309-01. "Functional Analysis of BRCA1 mutants". A.N.A Monteiro, P.I. 04/01/02 06/30/07. Direct costs awarded: $ 750,000.

The Julia Murtha Fund. "A novel approach to restoring BRCA1 function", A.N.A. Monteiro, P.I. 09/01/01 – 12/31/02. Direct costs awarded: $46,000.

U.S. Army Breast Cancer Research Program – Concept Award. "Restoring BRCA1 function with Antibiotics". A.N.A Monteiro, P.I. 04/01/01-03/31/03. Direct costs awarded: $50,000.

U.S. Army Breast Cancer Research Program BC000959. Postdoctoral fellowship for Blase Billack. (A.N.A. Monteiro, mentor) 07/01/01- 06/30/04. "Characterization and Use of Temperature-sensitive mutations of BRCA1 for the study of BRCA1 function. Total direct costs: $148,247.

New York State Health Research Science Board. Postdoctoral Fellowship *BC01-058.* "Functional Assay for BRCA1". Claudia Bernardi, Ph.D. (A.N.A. Monteiro, mentor) 01/01/02- 12/31/03. Direct costs awarded: $ 98,656.

CONCLUSIONS

By the end of the fourth year of our project we were able to achieve all of the goals defined in our proposal. We have made significant progress towards testing our hypothesis as well as generating important reagents that will be useful for other experiments. At this point, the experiments conducted here have refuted our initial hypothesis of a regulatory role for an intramolecular interaction in BRCA1. Nevertheless, the experiments revealed that a putative coiled-coil domain spanning exons 12 and 13 is important for transcription regulation.

More generally, the laboratory is now five years and during this time we were able to secure private, state and federal funding (including a NIH RO1) and to publish several articles on the biology of BRCA1. In conclusion, funding from the DoD grant was crucial for my establishment as an independent breast cancer researcher.

APPENDIX

ELSEVIER

BRCA1: the enigma of tissue-specific tumor development

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Recent evidence indicates that BRCA1, a gene product associated with breast and ovarian cancer susceptibility, is an important component of the cellular response to DNA damage. Despite being expressed ubiquitously in adult tissues, germline mutations in *BRCA1* predispose individuals to breast and ovarian tumors with only minor effects on the predisposition to cancer in other sites. The reason for this tissue specificity of BRCA1 carcinomas must be found if we are to understand fully why these tumors occur and to enable us to design efficient preventive and therapeutic regimens. Here I propose that tissue-specific rates of loss of heterozygosity in the *BRCA1* locus could contribute to tissue specificity in tumor development.

BRCA1, a gene associated with susceptibility to breast and ovarian cancer, was positionally cloned using linkage analysis of families with multiple cases of breast and ovarian cancer [1]. Several lines of evidence now suggest that the encoded gene product participates in the cellular DNA damage response, although its precise molecular functions have remained elusive [2]. Individuals carrying inactivating germline mutations in *BRCA1* display a highly penetrant cancer phenotype. The predisposing allele behaves as a recessive allele in somatic cells, and tumor initiation requires loss of the wild-type allele [1]. Although these mutations predispose individuals to several types of cancer, the substantial increase in cancer risk is mostly limited to carcinomas of the breast and ovaries [3,4]. This long-standing problem of tissue specificity has generated several speculative ideas, but there have been very few attempts to analyze these ideas systematically or to test them experimentally. Here, I discuss these ideas briefly and propose that an overlooked aspect of tumor development, tissue-specific rates of loss of heterozygosity, might also contribute to tissue specificity in *BRCA1* patients.

Differential tissue requirement

An idea that is often invoked to explain tissue specificity in cancer predisposition is that functional BRCA1 protein is required to maintain the normal phenotype in breast and ovary epithelia, but not in other tissues. Thus, loss of the wild-type allele in individuals carrying germline mutations in *BRCA1* would allow cancer development in breast and ovary but would not be expected to affect other tissues. Such a specific requirement could arise in at least three different but interconnected scenarios.

First, in unaffected tissues there might be functional redundancy whereby other proteins perform the same function as BRCA1, but in ovary and mammary gland these other proteins are not expressed at an adequate level. However, it has been argued that the severe embryonic lethality in *Brca1* $^{-/-}$ mice is inconsistent with this hypothesis [5], although it is possible, if unlikely, that cells only establish the redundant or overlapping pathways after embryonic development, once they have undergone terminal differentiation. Conceivably, comparisons of tissue-specific transcriptomes (the entire complement of RNAs transcribed from the genome) using bioinformatic approaches might provide an experimental system in which to identify these factors or rule out this possibility (e.g. human transcriptome map, http://bioinfo.amc.uva.nl/HTM-bin/index.cgi; SAGE genie, http://cgap.nci.nih.gov/SAGE; or Gene expression atlas, http://expression.gnf.org/cgi-bin/index.cgi).

Second, particular tissues might be more exposed to a certain type of damage that requires BRCA1 for its repair. This is exemplified by the tumorigenesis seen in xeroderma pigmentosum, which is associated with mutations in genes that are responsible for the removal of DNA lesions induced by UV light. Although this DNA repair pathway is present in several tissues, only the skin receives an enhanced mutational burden because of its exposure to UV light [6]. Interestingly, mammary gland tissue does have increased levels of carcinogenic estrogen metabolites that can adduct DNA [7,8], and these could be candidates for a breast-specific mutation burden. However, inactivation of *BRCA1* also predisposes men to breast cancer, albeit less than *BRCA2*, suggesting that *BRCA1*-linked cancer development does not necessarily depend on high estrogen levels [9]. Also, it could be that, instead of environmental or exogenous challenges, a major determinant for the generation of mutations is the high rate of proliferation in the mammary gland. This could account for the generation of single and eventually double-strand breaks due to stalled replication forks (replication stress).

Third, since its discovery BRCA1 has been implicated in a wide variety of functions including transcription regulation, mRNA processing, X chromosome inactivation, maintenance of genomic integrity, and ubiquitination [2,10–12]. Thus, it could perform specific functions in

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certain tissues with the function performed in breast and ovarian tissues being the only one relevant for tumor suppression. One such possibility would be to modulate the activity of estrogen receptor α (ERα) [13,14]. Transfection experiments in cancer cell lines and *Brca1* $^{-/-}$ mouse embryo fibroblasts suggest that BRCA1 can mediate ligand-independent repression of the receptors for estrogen and progesterone. These results led to the idea that absence of functional BRCA1 might result in unregulated activation of ERα and therefore excess proliferation [13,14]. Because these effects would be felt in tissues that are targets of hormonal regulation such as breast and ovary, they could explain tissue specificity. Intriguingly, analysis of BRCA1 tumors has revealed that these tumors are mostly ERα-negative [15]. It remains unclear whether these tumors originate from ERα-negative cells or whether receptor expression is lost during tumor development. Preliminary evidence from a breast cancer prevention trial indicates that cancer risk was not significantly reduced by the use of tamoxifen, an anti-estrogen, in women carrying mutations in *BRCA1* [16]. These results suggest that either the tumors initiate from ERα-negative cells, or that loss of ERα is an early event in tumor development. Either interpretation argues against a major role for ERα regulation by BRCA1 in tumor development. Also, because the role of estrogen in ovarian carcinogenesis has not been established, this explanation is not readily applicable to ovary cells. In any event, this mechanism could have a role on cancer progression, but it is unlikely to constitute a rate-limiting step in the absence of any other genetic change.

Delayed apoptotic response

Recently, Elledge and Amon proposed a hypothesis to explain BRCA1 tissue-specificity by suggesting a delayed apoptotic response in breast and ovary [5]. It is based on the idea that loss of BRCA1 function leads to apoptosis or severe proliferation defects in tissues other than breast and ovary, therefore preventing the accumulation of additional mutations required for tumor formation. Indeed, evidence derived from mouse models indicates that *Brca1* is an essential gene and its loss is incompatible with embryonic development [17]. According to this idea, cells undergoing loss of the wild-type allele would be eliminated rapidly unless tissue-specific factors could suppress or delay lethality. It is proposed that breast and ovary epithelial cells survive for longer periods in the absence of BRCA1, although the basis for this difference is not known. In this case, temporary suppression of lethality in breast and ovary cells lacking BRCA1 would allow sufficient time to accumulate additional mutations required for oncogenesis. The balance between proliferation and apoptosis is tightly maintained in the mammary gland, and cells undergo apoptosis after each estrogen cycle, suggesting that at least some cells are susceptible to apoptosis [18] but also indicating that the apoptotic response might be controlled by tissue-specific factors. Recent progress in identifying genes involved in the apoptotic response coupled to refinement of the mammary gland transcriptome should allow a systematic analysis of factors involved in this response.

Differential loss of heterozygosity

In the case of tumor suppressor genes, the canonical rate-limiting step of tumor initiation (loss of the wild-type allele) can be viewed as dependent on two classes of event that could lead to tissue-specific tumor initiation: proximal events, including the tissue-specific mechanisms by which a gene is inactivated (genetically or epigenetically); and distal events, which reflect the different biochemical outcomes of lack of that particular gene product in a specified tissue (Fig. 1). According to this arbitrary classification, the hypotheses mentioned in the previous paragraphs are concerned with distal events and overlook the possibility that tissue specificity might also be determined by proximal events; that is, tissue specificity for BRCA1 tumors might reflect higher rates of loss of the wild-type allele in breast and ovary than in other tissues (Fig. 1).

Very little is known about the mechanisms of loss of heterozygosity (LOH) in epithelial tumors in general, and less so in breast and ovarian carcinomas. Our current knowledge of possible mechanisms derives to a large extent from classic studies of retinoblastomas [19,20]. Nondisjunction with chromosome loss or with reduplication, interstitial and terminal deletion, gene conversion, mitotic recombination between homologous chromosomes and translocations are several mechanisms by which a cell might undergo LOH.

Mechanisms of LOH are tumor- and chromosome-specific, so any discussion on the subject is speculative until we know the pattern of LOH in *BRCA1* tumors [20,21]. Among the different mechanisms, mitotic recombination (MR) has a key role in tumorigenesis and seems to be major cause of LOH in mice [22,23]. For example, mice lacking the gene associated with Bloom syndrome, *Blm*, display cancer predisposition and an elevated rate of MR raising the possibility that LOH resulting from MR underlies the tumor susceptibility, although this possibility has not been formally tested [24]. In the following discussion, I focus primarily on MR-derived LOH.

The human *BRCA1* gene (24 exons) is unusual in its high content of *Alu* sequences with ~42% of the 81-kb sequence versus 10% in the entire genome [25]. It has been proposed that these sequences mediate recombination during meiosis promoting deletion of large portions of the *BRCA1* and are at the origin of several germ-line mutations [26]. Could an analogous mechanism occurring during mitosis account for the loss of the wild-type allele? Is there any evidence to suppose that MR rates vary in different tissues? And if so, would the rates of loss be higher in breast and ovary than in other tissues?

Experimental evidence based mostly on studies of somatic LOH at the heterozygous *APRT* locus, in which cells undergoing LOH become resistant to 2,6 diaminopurine, support the notion that MR rates, at least in mice, vary in a tissue-specific way [22,27]. Additional support for tissue-specific mutation spectrum and frequency come from data showing that distinct mechanisms lead to organ-specific genome deterioration and dysfunction in old age [28].

In human lymphocytes, there is interindividual variation in MR rates ranging over more than two orders of

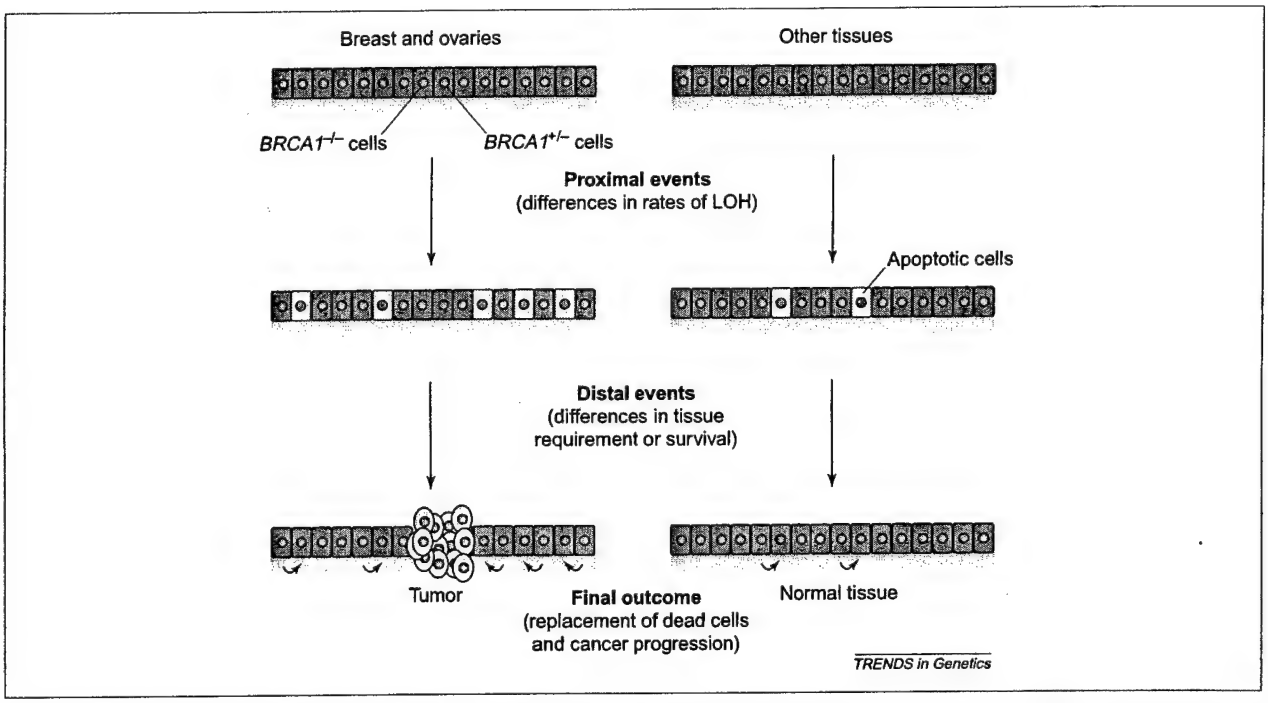

Fig. 1. The contribution of proximal and distal events to tissue-specific cancer development in individuals carrying germline mutations in *BRCA1*. Individuals carrying one wild-type copy and one mutated copy of *BRCA1* are predisposed primarily to breast and ovarian cancer, but show only minor effects on cancer risk in other tissues. BRCA1 tumor specificity could depend on rates of loss of heterozygosity (LOH) in breast and ovary (top left) that might be higher than in other tissues (top right) as indicated by the number of cells with red nuclei (*BRCA1*$^{-/-}$). Following loss of the wild-type allele, development of cancer (bottom left) might also depend on events that will determine whether a cell with inactivated *BRCA1* will become a cancer cell (bottom left; round gray cells with red nuclei) or be eliminated (middle, transparent cells) and replaced with normal cells (blue arrows).

magnitude, leading some authors to propose that it could be the basis for differences in lifetime risk of cancer [29]. It is tempting to speculate that such variation could also help explain the incomplete penetrance of *BRCA1*. Considering that breast cancer predisposition affects primarily women, it is interesting to note that rates of MR, as is the case in meiotic recombination, are significantly higher in females than in males [29].

The determinants for mitotic recombination and its frequency in mammals are not well established, but it is conceivable that a basis for such differences could be the tissue-specific chromatin environment at a particular locus. Exposed repetitive sequences would presumably be more prone to inter- and intra-chromosomal (causing interstitial deletions) recombination. One possible explanation for a tissue-specific chromatin environment might derive from differential transcription rates at the *BRCA1* locus. Although a variety of cells derived from adult tissues express BRCA1 in culture, tissue expression of BRCA1 *in vivo* seems to be restricted. Northern blots show that *BRCA1* transcripts are most abundant in testis and thymus, also present in breast and ovary, but are almost undetectable in other tissues [1]. Analysis of preliminary experiments from the human transcriptome map (http://bioinfo.amc.uva.nl/HTM-bin/index.cgi), for example, suggests that *BRCA1* transcripts are present in breast and kidney but not in colon. Moreover, *Brca1* expression is modulated during postnatal development of mammary gland, with higher levels during pregnancy, raising the possibility that the chromatin environment at the *Brca1* locus might differ during certain stages of development [30]. Under these circumstances, it is intriguing that although parity has been associated with reduced risk of breast cancer in general, this is not the case for carriers of *BRCA1* mutations [31]. In these cases, each pregnancy seems to be associated with increased risk [31]. Although speculative, this model generates several testable predictions.

First, if the differential susceptibility to recombination derives from a more relaxed chromatin environment, the *BRCA1* genomic locus should be more sensitive to DNase I or micrococcal-nuclease digestion when isolated from immunopurified mammary gland cells (e.g. from reduction mammoplasty) than from other tissues. This would reflect a tissue-specific chromatin structure.

Second, if the major determinant for mitotic recombination relevant for BRCA1 is the presence of *Alu* sequences, heterozygous mice will be less susceptible to the loss of the wild-type allele than their human counterparts (*Alu* sequences are found in extremely high copy number only in primate genomes). That is consistent with the rarity of mammary tumor development in *Brca1* $^{+/-}$ mice. To test this idea one could employ a strategy that has been developed to replace the mouse allele with the human *BRCA1* gene (including its *Alu* sequences) [32]. In this case, heterozygous mice with humanized *BRCA1* alleles (one wild-type and one inactivated allele), and therefore containing *Alu* sequences, might display higher incidence of mammary tumors than heterozygous (*Brca1* $^{+/-}$) mice carrying murine alleles.

Third, genetic divergence suppresses mitotic recombination [27]. Therefore, I predict that the penetrance of a particular mutation will be lower in more-diverse populations and higher in less-diverse ones. Unfortunately, this could be extremely hard to verify given the likely confounding contribution of modifier genes to penetrance.

As stated by Elledge and Amon on the subject of BRCA1 tissue specificity [5], all the present hypotheses are valid, with no overwhelming evidence for or against any of them. Many of the concepts underlying these various hypotheses are not mutually exclusive, and tissue specificity is likely to be explained by a combination of several factors. If we take an evolutionary view of tumor development, the outcome will be dependent on the amount of variability generated (LOH) as well as on the rate of fixation of these changes and on the selective advantage they confer (survival from apoptosis). This implies that both classes of event (proximal and distal) are important in the maintenance of the tumor phenotype. For example, the preferential LOH could mean that breast cells are more prone to lose the *BRCA1* locus and a differential apoptotic response could mean that these losses are more likely to be maintained in breast or ovary leading to accumulation of more mutations and a quicker progression to cancer.

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ONLINE MUTATION REPORT

Absence of constitutional *H2AX* gene mutations in 101 hereditary breast cancer families

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Several genes involved in the DNA damage response and in maintaining genomic stability have emerged as breast cancer susceptibility genes. These include *BRCA1* and *BRCA2*, as well as other genes with smaller contributions to breast cancer aetiology, such as *TP53*, *CHEK2*, and *ATM*. Germline mutations in *BRCA1* and *BRCA2* increase sensitivity to DNA damage and decrease cellular capacity to repair double strand DNA breaks through homologous recombination.[1–3] Interestingly, DNA damage induces activation of ATM (ataxia-telangiectasia mutated), which performs a central role in relaying signals that orchestrate DNA repair.[4] People with heterozygous nonsense mutations in *ATM* appear to display increased susceptibility to breast cancer.[5–7] Besides rapidly phosphorylating BRCA1,[8] activated ATM also phosphorylates p53 and CHEK2 (CHK2, hCDS1), which have been implicated in breast cancer predisposition. Germline mutations in *TP53* or *CHEK2* cause Li-Fraumeni syndrome, a multiple cancer phenotype syndrome, which features early onset breast cancer.[9 10] Recently, it was found that germline mutations in *CHEK2* also increase the relative risk for breast cancer outside the Li-Fraumeni syndrome.[11 12] Given the emerging relationship of impaired DNA damage response and breast cancer susceptibility, we hypothesised that other genes in this pathway might be candidate cancer susceptibility genes.

Histone *H2AX* (*H2AFX*, OMIM 601772) is such a candidate gene. H2AX is a minor variant of the highly conserved histone H2A that is part of the histone octamer in the core of the nucleosome.[13] It differs from H2A by having a longer carboxy-terminal tail that contains an SQE motif, a consensus site for phosphorylation by PI3K related kinases such as ATM, ATR, and DNA-PK. Following DNA damage, ATM phosphorylates H2AX at serine 139 (part of the SQE motif)[14] and phosphorylated H2AX (γ-H2AX) seems to localise specifically at sites of damage.[15 16] More importantly, several proteins involved in DNA repair including BRCA1, BRCA2, Rad51, and Mre11 are recruited to sites of γ-H2AX.[16] Two other independent lines of evidence derived from model organisms support the notion that H2AX plays an important role in the DNA damage response and in chromosomal stability. Phosphorylation of S129 of *Saccharomyces cerevisiae* H2A (homologous to S139 in human H2AX) is necessary for efficient processing of DNA repair and is proposed to cause alteration of chromatin structure that facilitates repair.[17] Mice lacking *H2AX* are sensitive to radiation, are growth retarded, and their cells display high levels of ionising radiation induced chromosomal instability.[18 19] Thus, although the exact function of H2AX is still unknown, it is clear that it plays a role in the DNA damage response.

To establish if germline *H2AX* mutations are present in breast cancer families, DNA samples were obtained from 101 unrelated breast cancer patients.

Key points

- Several genes whose products participate in the cellular response to DNA damage have emerged as breast cancer susceptibility genes. These genes include *BRCA1*, *BRCA2*, *ATM*, *CHEK2*, and *TP53* and germline mutations in these genes increase the predisposition to breast cancer.
- We hypothesised that other genes in the DNA damage response pathway might also be breast cancer susceptibility genes. The gene for histone *H2AX* is such a candidate. Its product is an early substrate of ATM kinase activity following DNA damage. Phosphorylated H2AX (γ-H2AX) colocalises with DNA breaks, and other proteins involved in DNA repair such as BRCA1, Rad51, and Mre11 are recruited to γ-H2AX sites.
- We screened subjects from 101 families with hereditary breast cancer by direct sequencing of *H2AX* coding sequence. We did not detect any mutations or sequence variants in this sample, suggesting that it is unlikely that germline mutations in *H2AX* play a major role in hereditary breast cancer.

METHODS AND RESULTS

Each of these patients was from a family with three or more cases of breast cancer. These families were selected because they have previously been tested for the presence of germline mutations in *BRCA1* and *BRCA2* using the protein truncation test (PTT) and no mutations were found. There were, on average, 4.2 cases of breast cancer per family (range 3 to 11) with an average of 3.0 cases of breast cancer in first degree relatives per family (range 2 to 9). Seventeen of the families also contained cases of ovarian cancer. For each family, a single patient affected with breast cancer was studied, with a mean age of diagnosis of 47 years (range 24 to 72 years).

The *H2AX* gene contains a single exon with 432 nucleotides. The coding sequence of the *H2AX* gene was evaluated by direct sequencing of a 561 bp fragment amplified using the following primers: F5′-CGTCTGTTCTAGTGTTTGAGC-3′ and R5′-TGAGGGCGGTGGTGGCCCTTAA-3′. No mutations or sequence variants were found in the 101 patients.

DISCUSSION

Our results suggest that germline *H2AX* mutations are unlikely to be common in families with familial breast cancer. Furthermore, the absence of polymorphic variation in this gene precludes the possibility that missense variants within the coding region of *H2AX* are associated with breast cancer risk in the population as a whole. It is, of course, possible that rare disease causing mutations in *H2AX* exist, and these might be uncovered in a larger study.

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SPECIFICITY IN SIGNALING BY c-YES

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1. ABSTRACT

c-Yes and c-Src are the two most closely related members of the Src family of nonreceptor tyrosine kinases. Although there is much evidence to support redundancy in signaling between these two kinases, there is also a growing body of evidence to indicate specificity in signaling. In this review, we summarize c-Yes, its potential functions and its ability to modulate signals that are distinct from c-Src.

2. INTRODUCTION AND SIGNIFICANCE

p62 c-Yes and p60 c-Src are two of the nine members of the Src family of non-receptor tyrosine kinases (c-Src, c-Yes, Fyn, Lyn, Lck, Hck, Blk, Fgr, and Yrk). Src family kinases are linked by a common structural architecture, each with four Src homology domains (SH1-4) and a Unique domain. The Src family kinases have been implicated in signaling pathways that regulate a vast array of cellular processes, including cytokine and growth factor responses, cytoskeleton dynamics, cell proliferation, survival and differentiation (1). While c-Src has been extensively studied, comparatively less is known of the biological functions of c-Yes. c-Yes and c-Src are two of the most widely expressed and homologous members of the Src family. Both kinases are present in many of the same tissues and are activated in response to many of the same stimuli. The frequent activation of c-Src and c-Yes in human cancers, coupled with the tumorigenic potential of their viral homologues, v-Src and v-Yes, suggests that they may contribute to the onset or progression of the malignant phenotype. Thus, c-Src and c-Yes represent potential targets for rational drug design. In order to optimize the value of these kinases as therapeutic targets, it will not only be important to understand the biological functions of c-Yes and c-Src, both in normal and cancerous tissues, but also to understand how they are capable of sending specific signals. As the biology and functions of c-Src have been extensively studied and reported, this review will focus on the functions of c-Yes and how c-Yes may be able to participate in specific signaling pathways. Special emphasis will be on differences between c-Yes and cSrc signaling.

3. V-YES AND V-SRC

The Yes kinase was originally discovered as the oncogenic protein encoded by the Yamaguchi 73 and Esh sarcoma viruses (2-6). Initial interest in v-Yes arose as a result of its similarity to v-Src. Both oncoproteins are tyrosine kinases capable of inducing sarcomas in chickens and transforming fibroblasts in culture (5,7). v-Src and v-Yes are 82% similar, with particularly strong homology

Figure 1. Anti-phosphotyrosine staining in c-Yes overexpressing MDCK cells. Overexpression of c-Yes correlates with increased cellular tyrosine phosphorylation in distinct cellular structures that resemble focal adhesions. The cYes oncoprotein was overexpressed in MDCK cells, fixed and labeled with anti-phosphotyrosine (green).

(90%) within the tyrosine kinase domain (8). The amino acid similarity between the two kinases allows immunorecognition of v-Yes by monoclonal antibodies raised against v-Src (9), and the two proteins release overlapping subsets of tyrosine-phosphorylated peptides upon tryptic digestion and two-dimensional electrophoresis (10). In addition to their structural homology, biochemical and functional similarities have been observed between v-Src and v-Yes in chicken embryo fibroblast culture. Both proteins fractionate with the detergent-insoluble cytoskeleton and localize to focal contacts, where the cell and extracellular matrix meet (Figure 1) (11,12). Many of the same proteins are phosphorylated in cells transformed by either v-Src or v-Yes (13,14). v-Src and v-Yes also appear to utilize shared downstream signaling pathways, as both proteins induce activation of PI3 kinase and require the activator protein 1 (AP-1) transcription factor complex (composed of heterodimers of various isoforms of the c-Jun and c-Fos transcription factors) for cell transformation (15,16).

Homology between v-Src and v-Yes can be extended further to include the mechanisms by which these kinases have been rendered transformation-competent. Both kinases are constitutively active due to loss or replacement of sequence in their carboxy-termini (17). Src family kinases contain a conserved tyrosine residue (Tyr^{527} in c-Src, Tyr^{535} in c-Yes) in their carboxy-terminal tail sequence. This tyrosine residue is phosphorylated by members of the Csk (C-terminal Src kinase) family and forms an intramolecular interaction with the SH2 domain (18). The SH2/tail interaction forms the linchpin of the "closed" or "assembled" conformation (19,20). The SH3 domain contributes to the stability of the closed conformation through formation of an intramolecular interaction with the kinase linker, a short stretch of amino acids bridging the SH2 and kinase domains (19,20). In the assembled state, the conformation of the kinase active site is such that ATP and substrates are prevented from entering, resulting in repressed kinase activity (21). Although recent evidence indicates that autophosphorylation within the active site (Tyr^{416} in c-Src, Tyr^{424} in c-Yes) may allow kinase activity in the presence of phosphorylation at the regulatory tyrosine (21,22), Src family kinases must be released from the closed conformation in order to achieve their full transforming potential. The ability of v-Src, v-Yes, Src^{527F} (in which Tyr^{527} is mutated to Phe), and various SH3 and SH2 domain mutants of c-Src to transform cells illustrates this point (17,23,24).

4. C-YES EXPRESSION PATTERNS: COMPARISON AND CONTRAST WITH C-SRC

Phylogenetically, c-Yes expression has been confirmed in simple vertebrates such as Xenopus (25) and teleost fish (26). Although there is a single report of a c-Yes homolog in drosophila (27), this observation was not verified. The potential absence of c-Yes from Drosophila is interesting given that there is a c-Src ortholog present in fly (28-30). Thus, it is possible that c-Yes may have evolved along a vertebrate line, although this has not been examined in great detail. The Xenopus and teleost sequences, as well as the avian, murine and human sequences of c-Yes have been deposited in the GenBank database. The accession numbers for c-Yes sequences in GenBank are: Rat (BAB21451), Xenopus (P10936), human (P07947), chicken (P09324), dog (Q28923), mouse (Q04736) and fish (P27447).

The YES gene was discovered as the cellular homologue of the v-*yes* oncogene (31-33). Initial reports localized the YES gene to chromosome 18 at locus q21.3 in humans (34-35). However, a refinement of this observation was reported via sequencing of YAC's, which indicated that the YES gene localizes nearby, to chromosome 18p11.32 (36). There does appear to be a pseudogene for YES, sometimes referred to as c-*yes*-2, found on chromosome 22q11.2 (37). Interestingly, the c-Yes related kinase, Yrk, has been reported to be present only in fowl (38). The significance of c-*yes*-2 is unknown; however, it may be noteworthy that c-yes-related sequences may be present as "fossils" in the genome. The YES gene encodes a 62 kDa tyrosine kinase that displays extensive similarity with the c-Src protein and, like c-Src, is expressed across a wide range of normal cells and tissues (39). c-Yes expression is found in epithelial tissues (lung, kidney, gastrointestine, liver, skin, etc.), connective tissue, vascular endothelial cells, and smooth muscle; expression levels are particularly high in platelets, neurons (particularly Purkinje cells of the cerebellum), and spermatid acrosomes (35,40-45). While c-Src is also expressed in many of these tissues, the expression patterns of the two proteins are not identical, thus providing evidence that c-Yes may perform unique functions. Mapping of the c-*yes* promoter region revealed a sequence that was reminiscent of other oncogenes and likely dictates its broad expression pattern (46). Gessler and Barnekow observed that the c-*yes* and c-*src* genes were differentially expressed during chicken embryogenesis

(47). In these studies, c-*src* message was expressed at high levels in brain throughout embryogenesis and displayed an age-dependent decrease in muscle tissue. Message levels of c-*yes* were initially low in brain, muscle, and heart, but increased throughout embryogenesis. Bixby and Jhabvala noted differential developmental expression of c-Src and c-Yes in the brain (48). Peak c-Src expression was observed between days 10 and 12, followed by a gradual decrease in expression, whereas c-Yes expression did not peak until day 20 and remained at high levels into adulthood. Sudol *et al.* noted a similar age-dependent decrease in chicken cerebellar c-Src expression, in contrast to increased c-Yes expression (49). Collectively, these data imply that c-Src and c-Yes may perform different functions in the developing embryo and adult.

5. INVOLVEMENT OF C-YES IN CELLULAR SIGNALING PATHWAYS

The ability of v-Yes to induce cellular transformation suggested that c-Yes may be involved in the control of cell proliferation and cell shape changes associated with the transformed phenotype. However, the fact that many of the cells in which c-Yes is highly expressed are post-replicative (i.e. neurons, platelets) and fully differentiated suggests a possible role in differentiation or the facilitation of cell-specific processes. In fact, evidence exists for involvement of both c-Yes, and Src family kinases in general, in a variety of signal transduction pathways that regulate cell division, differentiation, survival, motility, adhesion, spreading, and vesicular transport (1). In serum-starved NIH 3T3 fibroblasts, serum stimulation results in activation of c-Yes, along with c-Src and Fyn, during the G_2/M cell cycle transition (50). c-Yes and c-Src are both activated during proliferation and differentiation of rat trophoblast cells (51). In keratinocytes, calcium treatment results in decreased c-Yes kinase activity, coinciding with a shift from cellular proliferation to differentiation (52,53). Upon calcium treatment of epithelial cells, c-Yes, c-Src, and Fyn localize to sites of cell/cell contact, where their kinase activity is necessary for disruption of cell/cell junctions (54,55). In cultured neuronal cells, c-Yes, c-Src, and Fyn are concentrated and activated in growth cones (56). c-Yes, like c-Src, is also activated in response to stimulation of receptor tyrosine kinases (PDGF-R, CSF-1-R, Neu, FGF-R, Sky, Flt 1) (57-62), G-protein coupled receptors (angiotensin II receptor, thrombin receptor, endothelin receptor) (63-65), cytokine receptors (oncostatin M, interleukin-11, GM-CSF) (66-68), the FcεRI receptor, and the polyimmunoglobulin (pIg) receptor (69-70). Both c-Src and c-Yes will induce phosphorylation of common substrates (13-14). In addition, both c-Src and c-Yes will bind to common substrates, such as p120GAP or p38 (71-72). Collectively, these data indicate that c-Src and c-Yes have common and overlapping signaling functions. As previously hypothesized, it is quite likely that both c-Src and c-Yes function as amplifiers of receptor signaling (73).

6. SPECIFICITY IN SIGNALING BETWEEN C-YES AND OTHER SRC FAMILY KINASES

While the above list represents what is likely to be only a sampling of the pathways in which c-Yes is involved, it illustrates the potential for diversity in the function of this kinase. However, as c-Src and other Src family kinases are activated in response to many of the same cellular signals, it is not always clear where c-Yes performs unique functions and where it functions redundantly with other Src family kinases. The answer appears to vary from system to system. c-Yes may function redundantly with c-Src and Fyn in serum-induced fibroblast cell cycle progression. Microinjection of antibodies immunoreactive against all three proteins induces a cell cycle block at the G_2/M phase transition, whereas microinjection of a c-Src-specific antibody does not interfere with cell cycle progression unless no other Src family kinases are expressed (50). c-Yes may also function redundantly with c-Src and Fyn in PDGF-receptor signaling (74-75).

The phenotypes of the Src family kinase knockout mice provide further evidence for shared functions between Src family members. Whereas c-*src* -/-, *fyn* -/-, and c-*yes* -/- individual knockout mice develop distinct phenotypes, they typically survive for extended periods after birth. The loss of both the c-*src* and c-*yes* genes, however, invariably leads to perinatal lethality (76). Mice lacking both the *fyn* and c-*yes* genes develop degenerative renal damage, leading to diffuse segmental glomerulosclerosis (76). Interestingly, mice harboring a disruption of the c-*yes* gene do not display significant abnormalities in the cells or tissues in which c-Yes is most highly expressed (platelets, neurons, and spermatid acrosomes), suggesting that c-Src and/or Fyn are capable of compensating for the lack of c-Yes activity in these cells.

Despite the evidence for functional overlap, data also exist that indicate c-Yes- specific signaling. Much of this data is obtained from studies of c-*src* -/- mice and cells derived from them. Several of these studies indicate that c-Yes is unable to compensate for c-Src in processes that are dependent on the dynamic regulation of the actin cytoskeleton. Despite the presence of c-Yes, osteoclasts from c-*src* -/- mice are unable to reabsorb bone, resulting in an osteopetrotic phenotype (77). This deficiency correlates with an inability of the osteoclasts to form membrane ruffles and actin ring structures (78). Additionally, cells from the c-*src* -/- mice fail to spread properly on fibronectin and demonstrate reduced motility (79,80). Neuronal cells derived from c-*src* -/- mice are deficient in neurite extension on NCAM-L1 (81). Mice lacking the c-*src* gene also fail to develop Middle T antigen-induced mammary tumors, whereas c-*yes* -/- mice develop mammary tumors at a normal rate (82). Despite the apparent deficiency of c-Yes in eliciting these actin-dependent processes, active variants of c-Yes are localized to detergent insoluble cytoskeletal fractions (11,191). It is possible that c-Yes may associate with and/or regulate other components of the cytoskeleton. Indeed, it was observed by Ciesielski-Treska *et al.* that c-Yes co-localizes specifically with vimentin intermediate filaments in amoeboid microglia (83). Interestingly, c-Yes associates with adherens junctions (54). Nusrat et al. demonstrated that the tight junction associated protein, occludins, uniquely associates with c-Yes and not c-Src (84).

Occludins are transmembrane proteins that regulate extracellular interactions in tight junctions. Activation of Raf-1 is associated with down-regulation of occludins expression (85). Interestingly, chimeric constructs of c-Yes/Src^{527F} that contain the c-Yes SH3 domain (Y3^{527F}) fail to activate Raf-1, while chimeric constructs that contain the c-Src SH3 domain can activate Raf-1 (191). These data are consistent with a role for activated c-Yes as a binding signaling partner for occludins, while activation of c-Src might be predicted to direct activation of Raf-1 and downregulation of occludins. Thus, it is tempting to speculate that activated c-Yes may play a key role in participating in the maintenance of tight junction interactions, whereas activation of c-Src is known to cause their dissociation.

The inability of c-Yes to function as a "molecular backup" for c-Src is not limited exclusively to regulation of the actin cytoskeleton. In rat aortic smooth muscle cells, c-Src is specifically necessary for angiotensin II-induced phosphorylation of p120 Ras GAP and p190 Rho GAP (86). In rat ventricular myocytes, c-Yes, c-Src, and Fyn are activated in response to endothelin, however, only c-Src is able to drive elevated transcription from the atrial natriuretic peptide promoter, a hallmark of hypertrophy in ventricular cardiomyocytes (65). c-Src and c-Yes also differ in their respective abilities to participate in the hypoxia response. Of the three ubiquitously expressed Src family members, c-Src exclusively is activated under hypoxic conditions (87). This is in agreement with recent data that indicates that Src^{527F}/c-Yes chimeras with the c-Yes SH4 and Unique domains are deficient in upregulation of Heme Oxygenase 1 expression, as normally occurs in response to cellular stresses, including hypoxia (88).

Specificity between c-Yes and c-Src is not limited to pathways in which c-Yes fails to compensate for c-Src. While both c-Yes and c-Src are able to induce phosphorylation of the adaptor protein Cbl when overexpressed in COS cells, only c-Yes is able to efficiently co-immunoprecipitate with Cbl (89). During trophoblast cell proliferation, c-Yes associates with several tyrosine-phosphorylated proteins that are not found in complex with other Src family kinases (51). c-Yes is inactivated in keratinocytes upon calcium and phorbol ester treatment (53). In this system, c-Yes inactivation correlates with association with unknown proteins of 110 and 220 kDa (53). In 3T3 L1 mouse pre-adipocytes, both c-Src and c-Yes are activated upon IL-11 stimulation; however, only c-Yes is detected in a receptor-associated signaling complex including JAK2, PP2A, and gp130 (67). In angiotensin II-treated pulmonary vein endothelial cells, of the three ubiquitously expressed Src family kinases, only c-Yes induces phosphorylation of and association with the calcium-sensitive kinase Pyk2 (90). Unfortunately, the importance of co-association between c-Yes and the proteins mentioned above remains unknown. However, they do illustrate that c-Yes is capable of forming distinct protein/protein interactions and is thus capable of initiating or participating in unique signaling pathways.

In addition to differences in intermolecular interactions, signaling specificity between c-Src and c-Yes has been observed at the level of kinase activation. c-Yes is the predominant Src family kinase activated upon engagement of the Fœ receptor in the TF-1 mast cell line (69) and upon stimulation of neutrophils with oncostatin M (66). By virtue of its localization to lipid raft fractions, c-Yes may be selectively involved in the renal tubular cell internalization of Shiga toxin (Stx), produced by *Shigella dysenteriae* and enterohemorrhagic *Escherichia coli*. c-Yes associates with the globotriaosylceramide Gb3, to which Stx binds. Association of Stx with Gb3 induces c-Yes-specific kinase activation (91); however, the importance of c-Yes activity for Stx entry was not determined. In rat liver epithelial cells, c-Yes and Fyn are specifically activated in response to angiotensin II (63). In this system, c-Yes and Fyn are essential for the induction of DNA synthesis and c-Fos expression, as both responses are inhibited by microinjection of antibodies against either protein (63). Both c-Src and c-Yes, but not Fyn, appear to be required for VEGF-induced vascular permeability in mice, as cells lacking either protein are deficient in this response (92). Finally, c-Yes specifically associates with the polyimmunoglobulin (pIg) receptor and is activated upon receptor engagement (70). c-Yes appears to be required for pIg receptor-mediated transcytosis, as mice lacking the c-*yes* gene are deficient in transcytosis of IgA (70). Examples of cellular signals uniquely associated with c-Yes and not c-Src are summarized in Table 1. Taken together, these results indicate that in normal cells, c-Yes is able to send unique and specific signals.

7. CONTRIBUTIONS OF THE C-YES FUNCTIONAL DOMAINS TO SIGNALING SPECIFICITY

As a member of the Src family, c-Yes shares the functional domain architecture common to all Src family kinases. As mentioned above, this consists of an SH4 motif responsible for membrane localization, a Unique domain, followed by SH3 and SH2 domains, the protein tyrosine kinases or SH1 domain and a carboxy terminal regulatory region (17). While the c-Yes functional domains, specifically, have not been studied extensively, a great deal has been learned about the roles of Src family functional domains in general, and this knowledge will be important in the elucidation of how the c-Yes functional domains are likely to contribute to signaling specificity.

7.1. SH4 motif

The amino terminal 7-14 amino acids, sometimes referred to as the SH4 domain (98) or SH4 motif, is a region necessary and sufficient for localization to cellular membranes. All Src family members contain a glycine in the second position from the amino terminus. This glycine is myristoylated co-translationally and targets Src family members to cellular membranes. While the addition of myristic acid is necessary for membrane localization, it is not sufficient (98). All Src family members, with the exception of c-Src and Blk, additionally undergo palmitoylation at one or more cysteine residues downstream of the myristoylation site (98). Palmitoylation occurs post-translationally, and potentially spontaneously, as cysteine-acylation has been shown to occur

Table 1. Functions uniquely associated with c-Yes and not c-Src

Functions	Ref
c-Yes binds to a unique 85-87 kDa tyrosine phosphorylated protein	93
c-Yes binds to occludins	84
c-Yes binds to CD36 in human platelets	94
c-Yes regulates CD46 tyrosine phosphorylation in response to N. gonorrhoeae infection of epithelial cells	95
In keratinocyte hemidesmosomes, c-Yes binds to integrin a6ß4.	96
c-Yes mediates Et-1 stimulated glucose transport in 3T3 L1 adipocytes	97
c-Yes co-immunoprecipitates with Cbl	89
In trophoblasts, c-Yes associates with proteins not found in complex with c-Src	51
In keratinocytes, c-Yes associates with a novel 110 kDa and 220 kDa protein, unlike c-Src	53
In mouse pre-adipocytes, c-Yes binds to JAK2, PP2A, and gp130, unlike c-Src	67
In angiotensin II-treated pulmonary vein endothelial cells, c-Yes induces phosphorylation of and associates with the calcium-sensitive kinase Pyk2	90
c-Yes specifically associates with the pIg receptor and is activated upon receptor engagement.	70
c-Yes appears to be required for pIg receptor-mediated transcytosis, as mice lacking the c-yes gene are deficient in transcytosis of IgA	70
Ca^{2+} induces downregulation of c-Yes.	52
Activated c-Yes fails to alter actin filament integrity in CEF	191
c-Yes fails to compensate for c-Src in c-src -/- cells	77

spontaneously in the presence of acyl-CoA *in vitro* (99). However, prior myristoylation is required for palmitoylation (100). It has been suggested that palmitoylation is a dynamic process and may serve as a means of regulating the sub-cellular localization of Src family members and their access to substrates (101). Palmitoylation targets Src family members to lipid rafts, regions of the plasma membrane rich in glycolipids, sphingolipids, cholesterol, and glycosylphosphatidyl inositol-linked proteins, that can be isolated by flotation on sucrose gradients (102,103). The importance of the SH4 domain in raft localization has not been demonstrated specifically for c-Yes but has been shown for other Src family members, including Lck and Hck (102). However, since c-Yes is palmitoylated and is found in lipid rafts (104,105), it is likely that palmitoylation also serves to target c-Yes to these membrane microdomains.

Several recent studies have revealed the importance of palmitoylation and lipid raft localization in Src family kinase signaling. A functional palmitoylation signal is required for the ability of Src family kinases to efficiently transduce signals from the T cell receptor and Fc receptors (106-108). It has been speculated that the presence of c-Yes in lipid raft fractions in MDCK cells may be indicative of a role for c-Yes in vesicular trafficking or proper sorting of GPI-linked proteins (104,105).

7.2. Unique Domain

Following the SH4 domain is the Unique domain, a 60 to 90 amino acid sequence that is completely heterogeneous across the Src family. Very little is known about the role of the Unique domain in c-Yes signaling, and due to the lack of sequence homology in this region, it is difficult to infer a function for the c-Yes Unique domain based upon what is known of other Src family kinases. c-Src is phosphorylated on serine and threonine residues in the Unique domain during mitosis, and these phosphorylation events correlate with elevated kinase activity (109-111). Upon stimulation of the PDGF receptor in fibroblasts, Fyn is phosphorylated on tyrosine residues in its Unique domain, via both autophosphorylation and PDGF receptor phosphorylation (112). As with the c-Src Unique domain, phosphorylation of the Fyn Unique domain also correlates with increased kinase activity. An *in vitro* autophosphorylation site was mapped to tyrosine 32 in the c-Yes Unique domain, however, the importance of this phosphorylation event remains unclear (113). Phosphorylation of Unique domain residues may serve to facilitate protein/protein interactions or to alter the global conformation of the protein in such a fashion that the inactive "closed" conformation is destabilized, thus increasing kinase activity. In addition to serving as a site of phosphorylation, the Unique domain may also direct protein/protein interactions. The Unique domain is responsible for association of Lck with CD4 and CD8 in T cells, and association of Lyn with the Fce receptor (114,115).

7.3. SH3 Domain

Located carboxy-terminally to the Unique domain are the SH3 and SH2, two modular protein-protein interaction domains. These domains direct c-Yes signaling in several ways. First, SH3 and SH2 domain binding partners disrupt the intramolecular interactions that maintain the protein in the assembled or inactive conformation and enhance the accessibility of the kinase active site for ATP and substrates, thus increasing the specific activity of the kinase (116,117). Additionally, proteins binding to the SH3 or SH2 domains may be presented for tyrosine phosphorylation by c-Yes. Thus the SH3 and SH2 domains may contribute to signaling specificity through differential substrate selection and presentation for processive phosphorylation (118-122). Finally, stable complexes between c-Yes and SH3 or SH2 domain binding partners may serve as sites of activation or inactivation of downstream signaling pathways or target c-Yes to distinct sub-cellular locations (15).

The SH3 domain is approximately 60 amino

acids in length and directs protein/protein interactions through association with left-handed type II helical proline-rich sequences within SH3 domain binding partners (17,123,124). Domain swapping studies and *in vitro* binding assays have demonstrated overlapping ligand specificity between Src family SH3 domains but c-Yes SH3 domain swaps were not analyzed (120,125). Interestingly, studies utilizing phage display analysis have revealed subtle differences in the ligands selected by Src family kinase SH3 domains, including c-Src and c-Yes (126,127). In addition, differential protein binding has been demonstrated between the c-Yes and c-Src SH3 domains *in vitro* and *in vivo*. In comparison to the c-Src SH3 domain, the c-Yes SH3 domain is deficient in binding the 110 kDa actin filament associated protein (AFAP-110) *in vitro* (93). The significance of these observations is supported by *in* vivo studies in which Src^{527F}/c-Yes SH3 chimeras were unable to form a stable complex with AFAP-110, as demonstrated by a lack of co-immunoprecipitation between the proteins (93). Conversely, the c-Yes SH3 domain readily binds the 65 kDa Yes Associated Protein (YAP 65), a weak c-Src SH3 domain binding partner (128).

Another example of signaling specificity and the role of the SH3 domains is illustrated by the calcium-mediated activation and inactivation of c-Src and c-Yes, respectively (52,53). A systematic domain swap analysis between c-Src and c-Yes, including the Unique, SH2 and SH3 domains revealed that their respective SH3 domains are responsible for the opposite regulation by calcium (Monteiro and Hanafusa; unpublished data). In summary, despite redundant and overlapping functions with other Src family SH3 domains, the Yes SH3 domain is critical to confer signaling specificity.

7.4. SH2 Domain

Immediately following the SH3 domain is the SH2 domain. The SH2 domain is comprised of 90-100 amino acids that form a modular phosphotyrosine-binding motif (17). SH2 domain interactions are high affinity, with dissociation constants in the nanomolar range. The SH2 domain is comprised of a central β-barrel structure, flanked by two α-helices and a smaller β-sheet (129,130). The tertiary structure of the SH2 domain forms two binding pockets, the first for phosphotyrosine and the second for the pY+3 amino acid (129,130). SH2 domain binding specificity is dictated by the sequence of amino acids surrounding the phosphorylated tyrosine residue, particularly those on the carboxy-terminal side (131,132). The optimal c-Yes SH2 domain ligand sequence has not been specifically determined, however, all Src family SH2 domains appear to select the sequence pYEEI with maximal affinity (132). In support of the peptide-binding data, SH2 domain redundancy has also been demonstrated with regard to full-length protein binding. c-Src, Fyn, and c-Yes bind the same sites within the PDGF and CSF-1 receptors via their SH2 domains (116), and the c-Yes and c-Src SH2 domains appear to bind the same sites within the Neu protein (59).

Little data exists to suggest signaling specificity between Src family members at the level of the SH2 domain, however, it was recently noted that Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain display enhanced co-immunoprecipitation with an unknown phosphoprotein of approximately 87 kDa (pp87) (93). As the identity of this protein is currently unknown, the significance of complex formation between pp87 and the c-Yes SH2 domain is unclear. An 85 kDa tyrosine phosphorylated protein identified by Parker et al., as a co-immunoprecipitating protein with GPI-1 anchored protein from pancreatic acinar cells, in an immunecomplex which also included c-Yes and caveolin proteins, and was hypothesized to regulate endocytosis (133). It is has not been determined whether the reported pp87 and p85 proteins are related. These data suggest that c-Yes-specific signaling events may be generated through SH2 domain interactions. A recent study by Bradshaw *et al.* revealed that SH2 domain specificity between Src family members may arise as a result of differences within the phosphotyrosine binding pocket (134). It was noted in this study that the amino acid in the βC3 position of the pTyr-binding pocket contributes considerable energy to phosphotyrosine binding. c-Src contains a cysteine in this position, whereas all other Src family kinases, including c-Yes, contain a serine. Mutation of the cysteine to serine resulted in a four-fold increase in binding affinity for pTyr. This may have implications for the relative abilities of Src family kinase SH2 domains to associate with their cognate regulatory phosphotyrosines or intermolecular binding partners.

Homology modeling of human c-Yes on c-Src (PDB ID entry 2SRC) with SwissModel reveals the remarkable similarity between the two proteins (Figure 2) (190). The surface of the SH3 domain that recognizes the polyproline helix of binding partners is identically conserved, as is the core phosphopeptide binding surface of the SH2 domain. Additionally, the interdomain interfaces that stabilize the closed, inactive form of the enzyme are largely conserved. Most amino acid substitutions in c-Yes relative to c-Src are in surface residues that are not known to be important for regulation or binding interactions. However, there are differences in regions flanking the SH3 and SH2 binding surfaces that could reasonably be expected to alter binding specificity relative to c-Src. In particular, several substitutions in the SH3 domain cluster in or near the RT loop. These include E97 > T107 and T98 > E108 (in the RT loop) and T129 > N139, L120> E130, H122 > R132, and V111 > I121 which form a surface adjacent to the RT loop. The RT loop is often important for coordination of basic residues flanking the core PXXP recognition motif.

Additionally, there are a few differences in the SH3-linker-kinase and SH2 kinase interfaces which might alter the activation properties of the molecule. In Src, Gln 324 in the kinase domain hydrogen bonds with the linker; the corresponding residue in Yes is Pro 334, which cannot make an equivalent interaction. In the SH2 kinase interface, Glutamic acid residues 157 and 320 are both replaced by Aspartic acid in c-Yes, and Arg 156 corresponds to Lys 166 in c-Yes. Although these substitutions are conservative, they will alter the electrostatic environment of the interface and could

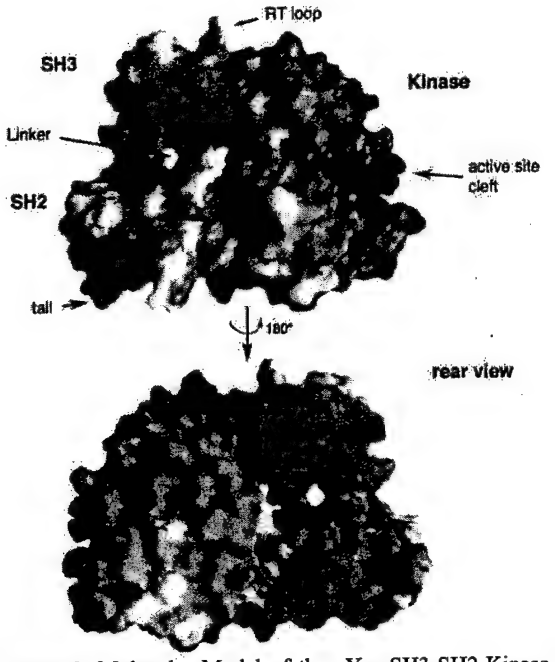

Figure 2. Molecular Model of the cYes SH3-SH2-Kinase domains. Domain organization of the auto inhibited Src kinase and analysis of surface residues that are different in Yes. The molecular surface representation of Src in the auto inhibited conformation (with Tyr 527 phosphorylated) is shown with the SH3 domain colored green, the SH2 domain orange, and the tyrosine kinase domain blue. The SH3 and SH2 domains pack against the "back" of the kinase domain, opposite the active site cleft, and help to lock the kinase in the inactive conformation. The linker (red), which connects the SH2 and kinase domains, packs between the SH3 and kinase domains and helps to maintain the inhibited conformation. Likewise, the phosphorylated C-terminal tail (purple) binds to the SH2 domain to lock the enzyme in the auto inhibited conformation. The portions of the surface formed by residues that differ in human Yes (from the corresponding residues in human c-Src) are colored yellow. Note that the differences are scattered among all domains, but are notably absent from the region of the kinase active site and the c-terminal tail. Divergent residues in and near the RT loop, which forms part of the recognition surface of the SH3 domain, may affect the binding specificity of the SH3 domain. Also, substitutions in the interfaces between the kinase and SH3 and SH2 domains may subtly alter the regulation of the kinase (see text).

therefore influence disassembly and activation of the closed form of the kinase. Additionally, substitutions within the hydrophobic core of the SH3, SH2 and Kinase domains may subtly affect their structure and therefore their binding properties as well. Thus, although there appears to be great conservation in the structure of the SH2 and SH3 domains of c-Src and c-Yes, there are sufficient differences to indicate mechanisms for differential interactions with distinct binding partners.

7.5. SH2/Kinase Linker

Following the SH2 domain is a short stretch of 15 amino acids that spans the gap between the SH2 and tyrosine kinase domains, referred to as the SH2/kinase linker. The primary function of the linker sequence is association with the SH3 domain in the closed conformation. In the closed conformation, the linker takes on the left-handed helical structure characteristic of SH3 domain binding partners. Mutations in this region can enhance kinase activity through destabilization of the closed conformation (135). Thus any contributions to signaling specificity made by the linker sequence would likely be reflected in the ease of enzymatic activation. Only one amino acid difference exists between the linker sequences of c-Yes and c-Src: the fourth position in the sequence is occupied by a serine residue in c-Src and a valine in c-Yes. The role of this amino acid in maintenance of the closed conformation has not been investigated, and thus, at this point no accurate assessment of its contribution to c-Yes signaling specificity can be made.

7.6. Tyrosine Kinase Domain

Following the linker sequence is the tyrosine kinase domain. This is the most highly conserved region across the Src family. As mentioned above, the kinase domains of c-Yes and c-Src are approximately 90% similar. The extremely high similarity between c-Yes and c-Src, and members of the Src family in general, in the kinase domain suggests that this region contributes little to signaling specificity. Differences in the optimal substrate sequences phosphorylated by Src and Lck have been demonstrated (136), however, these differences were subtle, and Src and Hck have been demonstrated to select the same peptide sequences for phosphorylation *in vitro* (137).

8. SPECIFICITY IN SIGNALING DICTATED BY C-YES FUNCTIONAL DOMAINS

There are differences in substrate specificity as modulated by the SH3 and SH2 domains between c-Yes and c-Src, as evidenced by different affinities for AFAP-110 and pp87 (or pp85). Differential protein binding between the c-Src and c-Yes SH3 domains was not unexpected as differences in SH3 domain ligand specificity, to the amino or carboxy terminal sides of the core PXXP motif, between Src family members, including c-Src and c-Yes, have been shown previously using *in vitro* peptide binding studies (126,127,138). The c-Src and c-Yes SH3 domains are very similar differing at only 11 amino acid positions (19,33); however, these amino acids have not been definitively identified as crucial for ligand binding. In lieu of the homology between the c-Src and c-Yes SH3 domains, it is of little surprise that ligand specificity detected between the two was subtle (LXXRPLPXΨP for Src, ΨXXRPLPXLP, where Ψ represents an aliphatic residue, and X represents any amino acid) (127). It was not determined if these differences in ligand specificity corresponded to actual differences in the ability of the c-Src and c-Yes SH3 domains to associate with full-length polypeptides, either *in vitro* or *in vivo*. The

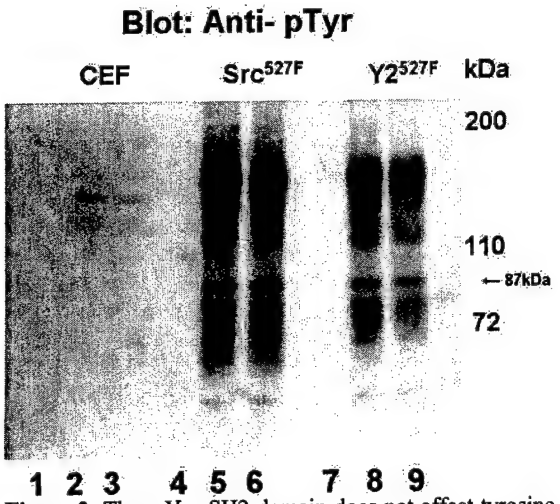

Figure 3. The c-Yes SH2 domain does not affect tyrosine phosphorylation of c-Src *in vitro* SH2 domain binding partners. (A) Mock-transfected CEF or cells expressing Src^{527F}, $Y3^{527F}$, $Y2^{527F}$, $Y32^{527F}$, or $Y4U32^{527F}$ were lysed in RIPA buffer, and five hundred µg of cell lysates were absorbed with GST, GST-SH2Src, or GST-SH2Yes bound to glutathione-sepharose beads. Bound proteins were eluted by boiling in Laemmli's sample buffer (LSB), and resolved by 8% SDS-PAGE. After transfer to PVDF membrane, proteins were blocked in 1% BSA/TBS-T and probed with a rabbit anti-phosphotyrosine antibody. Results are shown for mock-transfected CEF and cells expressing Src^{527F} and $Y2^{527F}$. Lanes 1, 4, 7 = GST; Lanes 2, 5, 8 = GST-SH2Src; Lanes 3, 6, 9 = GST-SH2Yes.

SH3 domains of c-Src, Fyn, and Lyn have previously demonstrated differential protein binding capacities *in vitro* (120), however this was not observed for the c-Src and c-Yes SH3 domains.

The data of Summy et al. (93) indicated that the differences in specificity between the c-Src and c-Yes SH3 domains are sufficient for differential protein/protein interactions both *in vitro* and *in vivo*. The individual amino acids responsible for ligand specificity, however, remain to be determined. Although systematic mutagenesis was not carried out to identify the individual residues responsible for imparting ligand specificity, they are likely to be localized to regions harboring non-conservative amino acid changes between the two SH3 domains. Glu^{97} and Thr^{98} in the c-Src SH3 domain, which correspond to Thr^{105} and Asp^{106} in the c-Yes SH3 domain, represent two non-conservative amino acid differences between the c-Src and c-Yes SH3 domains. These residues lie within the RT loop, a region that connects two of the core β sheets and contributes to ligand specificity (142,143), thus identifying these amino acids as potentially important for directing SH3 domain specificity between c-Src and c-Yes. Further studies, in which these residues in the c-Src and c-Yes SH3 domains are mutated, will be necessary to fully assess their importance for ligand specificity between the two proteins.

While differences in ligand specificity were expected between the c-Src and c-Yes SH3 domains, the evidence for differential protein binding between the c-Src and c-Yes SH2 domains was a surprise. *In vitro* binding assays indicate that Src family kinases select the same cognate peptide ligand sequences (132). The c-Src and c-Yes SH2 domains in particular have been demonstrated to bind the same sites within their cognate ligands (59,144). While no obvious differences in ligand specificity were detected between the c-Src and c-Yes SH2 domains *in vitro* (Figure 3), an 87 kDa tyrosine-phosphorylated protein (pp87) was observed to co-immunoprecipitate preferentially with Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain. This is strongly suggestive that pp87 represents either a direct or indirect SH2 domain binding partner. Again, the question remains as to which amino acids dictate SH2 domain specificity between c-Src and c-Yes.

The SH2 domain is composed of two binding pockets separated by a large central β sheet (129,130). The two binding pockets accommodate phosphotyrosine and downstream residues within the cognate ligand respectively (129,130). In the case of Src family kinases, the second ligand-binding pocket is typically occupied by a hydrophobic amino acid three residues downstream of the phosphotyrosine (pY+3) (130). As phosphotyrosine binding is a conserved feature of all SH2 domains, and differences in the downstream binding pocket dictate ligand specificity between the various SH2 domain sub-classes, the pY+3 pocket initially seemed the more attractive candidate for directing specificity between Src family kinases.

There are 27 amino acid differences between the c-Src and c-Yes SH2 domains, however, the most notable non-homologous differences between the SH2 domains, within the pY+3 pocket, occur in the B helix, which forms the bottom of the pocket. These differences occur at Gln^{223}, Ala^{228}, and Tyr^{229} in Src^{527F}, corresponding to Lys^{231}, Lys^{236}, and His^{237} in c-Yes, respectively (amino acid numbers correlate with chicken c-Yes). However, the existing crystal structures of Src family SH2 domains (19,20) suggest that only His^{237} is likely to be directed toward the interior of the ligand-binding pocket (129). In a recent paper by Bradshaw *et al.*, it was reported that Cys^{185} in the c-Src SH2 domain phosphotyrosine pocket, occupied by serine in all other Src family kinases (Ser^{193} in c-Yes), imparts a reduced affinity for phosphotyrosine relative to serine (134). Differences in phosphotyrosine affinity may represent a mechanism for dictating ligand specificity between the c-Src and c-Yes SH2 domains, as a non-consensus SH2 domain binding partner may be more readily accommodated by a higher affinity for phosphotyrosine.

Differential phosphotyrosine affinity between the c-Src and c-Yes SH2 domains may also have implications for the relative abilities of these kinases to be activated in response to cellular signals. A higher affinity for phosphotyrosine may allow more efficient association between the phosphorylated tail and the SH2 domain, thus stabilizing the inactive, closed conformation. Interestingly, it was observed that c-Src/c-Yes chimeras with the c-Yes

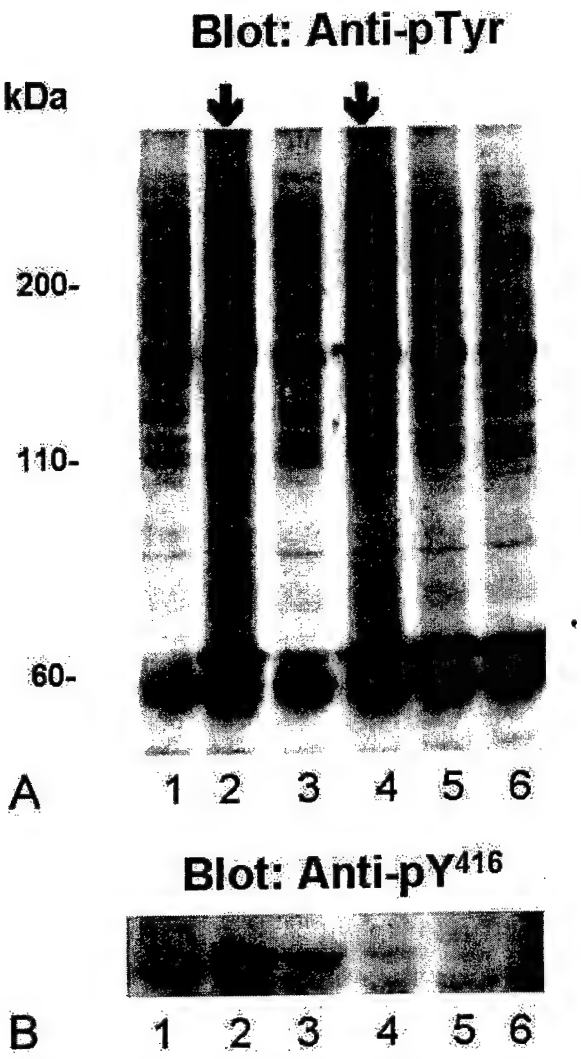

Figure 4. Induction of cellular phosphotyrosine and autophosphorylation of c-Src/c-Yes chimeras. Mock-transfected CEF or cells expressing c-Src, c-Yes, or c-Src chimeric constructs expressing c-Yes SH3 domain (Y3), c-Yes SH2 domain (Y2), both the c-Yes SH2 and SH2 domains (Y32), or the c-Yes SH4/Unique/SH3/SH2 domains (Y4U32) were lysed in RIPA buffer, and 30 µg of lysates were resolved by 8% SDS-PAGE, transferred to PVDF, blocked in 1% BSA/TBS-T, and probed with a rabbit anti-phosphotyrosine antibody. Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = c-Yes; Lane 4 = Y3; Lane 5 = Y2; Lane 6 = Y32; Lane 7 = Y4U32. (B). CEF, c-Src, Y3, Y2, Y32, or Y4U32 lysates were prepared for western blot analysis as described above and probed with a rabbit anti-phospho-Y416 antibody. Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = Y3; Lane 4 = Y2; Lane 5 = Y32; Lane 6 = Y4U32.

SH2 (Y2) domain induced much lower levels of cellular phosphotyrosine and autophosphorylation than corresponding constructs with the c-Src SH2 domain (Figure 4). Mutation of Ser^{185} to Cys in Y2, however, did not increase cellular phosphotyrosine or autophosphorylation of this kinase (Figure 5A).

In an effort to determine why c-Src/c-Yes chimeric constructs with the c-Yes SH2 domain displayed reduced levels of cellular phosphotyrosine and autophosphorylation, the possibility that the closed conformation was stabilized by an ionic interaction between a positive-charged amino acid in the c-Yes SH2 domain (His^{237}) and a negative-charged residue in the regulatory tail (Glu^{531} in c-Src, Asp^{539} in c-Yes) was explored. Again, however, mutation of these residues to uncharged amino acids (His^{237} to Tyr, as in c-Src and Glu^{531} to Gln) did not result in increased kinase activation, as evidenced by autophosphorylation (Figure 5A, leftl) and phosphorylation of the regulatory tyrosine Tyr^{527} (Figure 5B, right panel). It thus appears that additional residues are involved in directing specificity between the c-Src and c-Yes SH2 domains. Nevertheless, the results indicate that the c-Src and c-Yes SH2 domains may contribute to signaling specificity between these proteins and may play a larger role in directing signaling specificity between Src family kinases than previously believed.

What are the functional implications of ligand specificity between the c-Src and c-Yes SH3 and SH2 domains? The results to date do not suggest a strong influence on substrate selection. The tyrosine phosphorylation of AFAP-110 and other tyrosine-phosphorylated SH3 domain binding partners induced by $Y3^{527F}$ (a Src^{527F} chimera containing the c-Yes SH3 domain) was surprising given the inefficient co-immunoprecipitation and *in vitro* binding between these proteins. As mentioned above, SH3 domain interactions are necessary for Src-induced tyrosine phosphorylation of AFAP-110 and other SH3 domain binding partners (118,120,121). Mutations in either the SH3 domain binding site of AFAP-110 or the Src SH3 domain impair the ability of Src^{527F} to induce tyrosine phosphorylation of AFAP-110 (118,121). Due to the failure of the c-Yes SH3 domain to bind AFAP-110 efficiently *in vitro* or *in vivo*, a corresponding deficiency in the ability of $Y3^{527F}$ to induce AFAP-110 tyrosine phosphorylation was expected. This was not the case, however, as $Y3^{527F}$ and Src^{527F} induced comparable levels of AFAP-110 tyrosine phosphorylation (93). How are these seemingly contradictory results explained? One possible explanation is that the c-Yes SH3 domain, as opposed to a c-Src SH3 domain in which residues critical for ligand binding have been mutated, is capable of low-level association with AFAP-110. As seen by Summy et al., there is modest association of the c-Yes SH3 domain with AFAP-110 *in vitro*, and this correlates with inefficient yet still detectable co-immunoprecipitation between $Y3^{527F}$ and AFAP-110 in RIPA lysates. This relatively weak association between the c-Yes SH3 domain and AFAP-110 may allow a transient association between AFAP-110 and $Y3^{527F}$ that is sufficient to permit tyrosine phosphorylation of AFAP-110 but not detectable co-immunoprecipitation between the two proteins. Conversely, deletion of amino acids 92-95 in the Src SH3 domain may have abrogated any interaction with AFAP-110, resulting in significantly reduced tyrosine phosphorylation of AFAP-110 by $Src^{527F/dl92-95}$ (121).

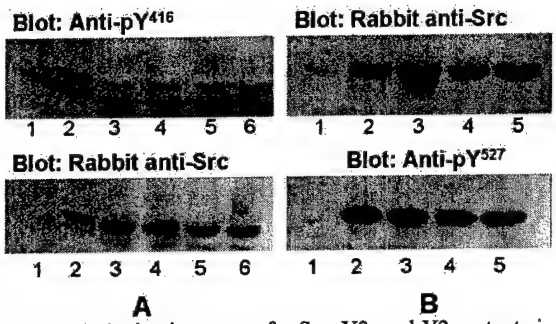

Figure 5. Activation state of c-Src, Y2, and Y2 mutants in CEF. Mock-transfected CEF or cells expressing c-Src, Y2, Y2S185C, Y2H229Y, or Y2E531Q were lysed in RIPA buffer, and 50 μg of lysates were resolved by 8% SDS-PAGE, transferred to PVDF, blocked in 5% milk/TBS-T, and probed with either a rabbit anti-phospho-Y416 antibody (A, top panel) or a rabbit anti-phospho-Y529 antibody (B, top panel). In the bottom panels, blots were stripped and re-probed with rabbit anti-Src, which reacts with a conserved carboxy terminal peptide sequence common to c-Src and c-Yes. (A) Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = Y2; Lane 4 = Y2S185C; Lane 5 = Y2H229Y; Lane 6 = Y2E531Q. (B) Lane 1 = CEF; Lane 2 = Y2; Lane 3 = Y2S185C; Lane 4 = Y2H229Y; Lane 5 = Y2E531Q.

The benefit of the more stable association between the c-Src or Src^{527F} SH3 domain and AFAP-110 remains unclear, as it does not result in a significant enhancement in tyrosine phosphorylation. Higher affinity association between the c-Src SH3 domain and AFAP-110 may allow formation of longer lived signaling complexes with distinct functions, including sub-cellular targeting, recruitment of signaling molecules, or alterations in the multimeric status of AFAP-110 itself. AFAP-110 has an intrinsic ability to bind actin filaments (146), and phosphorylation of AFAP-110 by PKCα activates the ability of AFAP-110 to induce actin filament bundling *in vitro* (95). Thus, AFAP-110 may play a key role in remodeling the actin filament cytoskeleton in response to cellular signals, as occurs during embryonic development, cell division, and metastasis of cancer cells. Src^{527F}/c-Yes chimeras with the c-Yes SH3 domain are capable of inducing actin filament remodeling in CEF comparable to Src^{527F}, thus it does not appear that stable association between Src^{527F} and AFAP-110 is a requirement for induction of actin filament rearrangement. However, it is noteworthy that $Src^{527F/dl92-95}$ exerts different effects on cells, whereby the cells are fusiform without the formation of actin filament rosettes (118). AFAP-110 association with active Src is also not sufficient to target Src to focal adhesion structures, as Fincham *et al.* demonstrated that mutants of Src that failed to localize to focal adhesions were still able to associate with AFAP-110 (147).

Regardless of the biological importance of stable association between activated c-Src and AFAP-110, the inefficient association of c-Yes with AFAP-110 would presumably preclude the involvement of c-Yes in these signaling pathways. Conversely, stable association between pp87 and Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain, and theoretically activated c-Yes itself, may allow these proteins to participate in signaling pathways that do not involve c-Src. This may occur, as described above for Src/AFAP-110 interactions, through sub-cellular localization or formation of c-Yes-specific signaling complexes. Unfortunately, in comparison to c-Src, little is known about the biological functions of c-Yes, and there are few intermolecular interactions between c-Yes and its cellular binding partners that have demonstrated definitive functional implications. Association of c-Yes with YAP65 may facilitate localization of c-Yes to the apical surface in airway epithelial cells (148), and it was recently demonstrated that recruitment of c-Yes to a signaling complex including β-arrestin 1 and the endothelin type-A receptor might allow c-Yes to participate in endothelin-stimulated glucose transport (97). The only phenotype reported thus far as a result of loss of the c-*yes* gene is a deficiency in transcytosis mediated by the pIg receptor (70). Expression of the Src^{527F}/c-Yes chimeras in c-*yes* -/- cells may prove useful in uncovering why c-Src is unable to compensate for c-Yes in pIg receptor function and what role c-Yes-specific binding partners may play in this pathway.

The affinity of the c-Src and c-Yes SH3 and SH2 domains for their cognate ligands also has implications for the activation of these kinases in response to cellular signals. The HIV-1 Nef protein is able to bind and activate Hck through SH3 domain interactions (117). Greenway and colleagues found that SH3 domain specificity between Src family kinases allows HIV-2 and SIV Nef to target the SH3 domains of c-Src and Fyn, as opposed to Hck (149). Baisden *et al.* demonstrated that a mutant of AFAP-110, in which the leucine zipper motif is deleted, is able to activate c-Src in an SH3 domain-dependent fashion, most likely through displacement of the intramolecular interaction between the SH3 domain and the kinase linker (150). The low affinity interaction between AFAP-110 and the c-Yes SH3 domain may be insufficient to permit AFAP-110-induced activation of c-Yes in response to cellular signals. The functions of AFAP-110, both downstream and upstream of c-Src, remain the subject of ongoing investigations by others. It remains possible that pp87 binding contributes to the activation of c-Yes in a similar fashion, however, this will be difficult to determine experimentally until pp87 is cloned and identified.

Thus, the known c-Src substrates p130 CAS and AFAP-110, as well as several unidentified tyrosine phosphorylated SH3 and SH2 domain binding partners, have not provided evidence for SH3 or SH2 domain-mediated substrate specificity between activated c-Src and c-Yes. While these results should not be over-interpreted to imply that SH3 and SH2 domain-mediated substrate selection between c-Src and c-Yes do not exist, the results imply that they may not be the primary means of generating signaling specificity. The SH3- and SH2-mediated specificity in protein binding observed in these experiments may contribute more to overall signaling specificity through differential signaling complex formation than through substrate selection.

In contrast to the differences in protein/protein interactions, differences in transcriptional activation and cell biological responses were primarily found to be due to differences in the Src^{527F} and c-Yes amino terminal regions, including the SH4/Unique domain. Chimeric proteins with the c-Yes amino terminal SH4/Unique/SH3/SH2 domains, $Y4U32^{527F}$, or $Y4U^{527F}$, which only contains the cYes SH4/Unique domain, were deficient in upregulation of the *heme oxygenase* 1 (HO-1) gene product (88) and induction of the morphological and cytoskeletal changes that occur concomitant with overexpression of constitutively activated c-Src despite the fact that they were fully active, as evidenced by anti-phosphotyrosine and anti-phospho-Y^{416} western blot analysis (191). The decreased HO-1 induction and failure of these chimeras to induce rearrangement of the actin cytoskeleton was attributed to the presence of the c-Yes amino terminus, as $Y32^{527F}$ (Src^{527F} chimera containing the c-Yes SH3/SH2 domains) was fully functional in regard to both of these processes. Both the SH4 and Unique domains were necessary in order to obtain these results, as chimeric proteins in which only the SH4 or Unique domain of Src^{527F} was replaced by that of c-Yes were unable to induce actin filament rearrangement and efficient upregulation of HO-1 expression in CEF.

In recent years, it has become clear that the amino terminal regions of Src family kinases do not simply localize these proteins to cellular membranes; they also regulate the compartmentalization of these kinases within cellular membranes. Specifically, it has been demonstrated that palmitoylation of Src family members dictates their inclusion into triton X-100 resistant membrane fractions, referred to as lipid rafts (103). These are formed by aggregates of bulky lipids, including sphingolipids and cholesterol, and glycosylphosphatidyl inositol (GPI)-linked proteins (151). Several acylated signaling proteins, including many Src family kinases, are recruited to lipid raft fractions by virtue of their long chain fatty acid modifications. Fatty acylation is not necessarily a requirement for inclusion into lipid raft fractions, however, as several transmembrane receptors localize to lipid rafts, including the PDGF and Fcε receptors (107,151).

Recruitment to lipid rafts is important for the participation of Src family kinases in several signaling pathways. The detection of c-Yes in caveolae, a sub-class of lipid rafts that are defined by the presence of caveolin, was among the first reports of Src family kinases in lipid rafts (43). The importance of c-Yes localization to caveolae, however, was not determined. Shenoy-Scaria *et al.* were among the first to observe that association between Src family kinases and a cellular binding partner was dependent on palmitoylation and localization to lipid rafts (102,105). At a functional level, co-localization of the Fcε receptor and Src family kinases in lipid raft fractions is essential for Fcε receptor signaling (107,108). In this system, it was demonstrated that raft localization serves as a means of achieving signaling specificity, as c-Src was unable to reconstitute Fcε receptor signaling unless a palmitoylation signal was included in the amino terminus (107,108). Palmitoylation-dependent signaling is also observed for Lck, which is unable to transmit signals from the T-cell receptor in the absence of palmitoylation (101).

The results obtained by Summy et al., (191) were particularly novel, in that the entire amino terminal region, including both the SH4 palmitoylation signal and the Unique domain, was required in order to achieve the differential effects on induction of Heme oxygenase I (HO-1) transcription and changes in the actin cytoskeleton. This is of note in that previous studies have traditionally reported the functions of the SH4 and Unique domains individually, with little consideration given to the possibility that these domains may act in concert. The heterogeneity of the Unique domain across the Src family renders it virtually impossible to assign a generalized function. Interestingly, computer-based secondary structure predictions of the Unique domains from different Src family kinases predict a relative conservation of structure (Flynn, unpublished observation). This could indicate that the Unique domains could have a conserved structure without conservation of sequence, not unlike that seen with PH domains. Lck associates with the CD4 and CD8 T cell co-receptors by virtue of its Unique domain (114,152), whereas the Unique domain of Lyn is responsible for mediating its association with the Fcε receptor (115). The Unique domains of Fyn, c-Src, and c-Yes harbor phosphorylation sites that demonstrate some correlation with activation, however, the full importance of these phosphorylations remains unknown (109-113). Bijlmakers and colleagues reported that the Unique domain of Lck is sufficient for membrane localization, however, the construct used in those studies included the Lck SH4 region (153).

Unlike the Unique domain, the function of the SH4 domain has been well established. Myristoylation, in conjunction with either palmitoylation or positive-charged amino acids, localize Src family kinases to cellular membranes (98). Palmitoylation then localizes Src family kinases to lipid raft fractions, allowing them to participate in signaling pathways originating in these membrane microenvironments. The partial requirement of the c-Yes SH4 domain for the inability of Src^{527F}/c-Yes chimeras to induce actin filament rearrangement and elevated HO-1 expression suggests that localization to lipid rafts is important in these results. However, the additional requirement for the c-Yes Unique domain complicates the issue. These results indicate that either the Unique domain participates in localization or that it, in conjunction with the SH4 domain, facilitates interaction with a protein or proteins that inhibit the ability of $Y4U32^{527F}$ and $Y4U^{527F}$ to influence signaling pathways that induce HO-1 expression and actin filament rearrangement. Alternatively, the c-Yes Unique domain, again in conjunction with the SH4 domain, may prevent interaction with a Src substrate or binding partner that is necessary for initiation of these signaling events. Regardless, it is clear that both the c-Yes SH4 and Unique domains prevent induction of HO-1 expression and actin filament rearrangements. Thus, evidence presented that indicate a synergistic effect of the SH4 and Unique domains in c-Yes

signaling could indicate that these two regions may actually function as one domain.

What do these results reveal about the biological functions of c-Yes and the signaling pathways it utilizes? Unfortunately, they suggest more about what activated c-Yes cannot do than what it can. However, they may also provide insight as to why c-Yes is frequently unable to compensate for the absence of c-Src in c-*src* -/- cells. The c-Yes amino terminal region is necessary and sufficient to inhibit the ability of Src^{527F}/c-Yes chimeras to participate in pathways that effect elevated induction of HO-1 and rearrangement of actin stress fibers into rosettes, lamellipodia, and filopodia. These results correlate well with data in the literature that indicate that c-Yes is unable to participate in related signaling pathways. Heme oxygenase 1 is an enzyme that cleaves the heme molecule into iron, carbon monoxide, and biliverdin (140,154). HO-1 is expressed in a wide range of tissues in response to diverse cellular stimuli, including hypoxia (140), heavy metals (155), Ras (156), MAP kinases (139,157), NFκB (139), Hypoxia-Inducible Transcription Factor 1 (HIF-1α) (158), and, Src activation (88). The ability of activated Src to induce HO-1 expression was not surprising in lieu of the fact that tyrosine kinase inhibitors block HO-1 induction in response to heavy metals (155) and given the ability of v-Src to induce HIF-1α expression (159). In fact, the reduced induction of HO-1 by $Y4U32^{527F}$ and $Y4U^{527F}$ may be due to a failure to induce expression of HIF-1α.

Chimeric proteins with the c-Yes amino terminus failed to induce activation of the PI3K/Akt pathway (191). Several recent studies have indicated that activation of PI3K and/or Akt are necessary for induction of HIF-1α (160-162). Failure to activate PI3K may prevent $Y4U32^{527F}$ and $Y4U^{527F}$ from efficiently activating an oxidative stress response pathway that involves HIF-1α expression and subsequent expression of HO-1. These results suggest that c-Yes, by virtue of its amino terminal region, may not participate in this oxidative stress response pathway. In agreement with this hypothesis, it was previously observed that c-Src, but not c-Yes, is activated in response to hypoxia, and results in expression of vascular endothelial growth factor, which is also induced in response to HIF-1α (87).

The inability of $Y4U32^{527F}$ and $Y4U^{527F}$ to induce actin cytoskeletal rearrangement and morphological changes is also in agreement with results reported previously. Cells lacking the c-*src* gene are deficient in several processes that are dependent on the dynamic regulation of the actin cytoskeleton. These include cell migration (80), cell spreading (79), neurite extension (81), membrane ruffle and ring formation (78), and osteoclast-mediated bone resorption (77). Recent results suggest that the c-Yes amino terminus may prevent $Y4U32^{527F}$ and $Y4U^{527F}$, and by extension activated c-Yes, from inducing remodeling of the actin cytoskeleton (191). Cells transfected with $Y4U32^{527F}$ or $Y4U^{527F}$ retained a normal CEF morphology, characterized by intact focal adhesions and long actin stress fibers. In contrast, cells expressing Src^{527F} and the other activated Src^{527F}/c-Yes chimeras displayed a fully transformed morphology and a repositioning of actin from stress fibers into rosettes and actin-based membranous motility structures, such as lamellipodia and filopodia.

9. C-YES IN CANCER AND DISEASE

The ability of v-Yes to induce cell transformation piqued interest in the study of the normal cellular homologue, as it was inferred that c-Yes might be involved in the onset or progression of human cancers. As with c-Src, several lines of evidence have in fact pointed to this possibility. The evidence is largely circumstantial, however, and consists primarily of observations that c-Yes is activated in several transforming, cancerous, and pre-cancerous conditions. Nevertheless, the data discussed below have laid the groundwork for future studies of c-Yes in human cancers. Both c-Yes and c-Src are frequently activated in human colon carcinoma cells and pre-malignant lesions of the colon (141,163-166). The degree of c-Yes and/or c-Src kinase activation in these cells correlates with their malignant potential (141,165). In colorectal carcinoma liver metastases, the kinase activity of either c-Src or c-Yes decreases relative to the parent tumor (167). However, activation of c-Yes in colon carcinoma liver metastases is associated with more aggressive disease and a poorer prognosis than c-Src activation (167). Interestingly, Park and Cartwright demonstrated that while c-Src activity increases during mitosis of human colon carcinoma cells, c-Yes protein expression and activation decrease (168). c-Yes protein levels and kinase activity are elevated 5-10 fold in malignant melanoma cells, in comparison to normal melanocytes, whereas c-Src activity and expression are unchanged (169). The c-yes proto-oncogene has also been reported to be amplified in human gastric cancer (170). c-Yes kinase activity correlates with the brain metastatic potential of melanoma cell lines, as the most highly metastatic melanoma cell lines display the highest levels of c-Yes kinase activity (171). c-Yes activity is further elevated upon stimulation with NGF or neurotrophin 3 (171). The neurotrophin-induced activation is specific for c-Yes, as c-Src activity is not affected. Despite the apparent dispensability of c-Yes kinase activity for Middle T antigen-induced murine breast tumors, the ability of Middle T to transform cells correlates well with its ability to associate with and activate both c-Src and c-Yes (172-177). While c-Yes activity does not appear to be necessary for transformation of fibroblasts by Middle T, it is important for Middle T-induced transformation of endothelial cells (82). In human breast cancer cells, overexpression of members of the EGFR family, specifically erbB-2 (Neu), is associated with poor clinical prognosis. When expressed in mice, under control of the mouse mammary tumor virus (MMTV) promoter, the Neu protein induces transformation of breast epithelial cells. In this system, the Neu protein associates with and activates both c-Src and c-Yes through SH2 domain interactions (59). Moasser *et al.* demonstrated up-regulation of c-Src and/or c-Yes kinase activity in a panel of human breast cancer cell lines, and inhibition of Src family kinase activity in this system resulted in cell cycle arrest during mitotic prophase (178). Together, the above data illustrate

that not only is c-Yes activated in select human cancers, but its contributions to the transformed phenotype may differ from those of c-Src. Furthermore, the differential activation of c-Yes and c-Src in some human cancers suggests that it may be beneficial to selectively target these kinases individually using rational drug design. In order to accomplish this, however, it is necessary to understand how these kinases send specific signals.

The results in this review raise questions about the relative contributions of c-Src and c-Yes to the onset and progression of human cancers. The transition of a cell from the normal to the cancerous state is a multi-step process, and while the biochemical pathways utilized to achieve full transformation may vary, there are certain phenotypic changes that are almost universally present. The experiments outlined in these studies directly and indirectly touch on four of these: cell proliferation, resistance to apoptosis, rearrangement of the cytoskeletal architecture, and the ability to survive in the face of oxidative stress. Cancerous cells are characterized by unchecked cell division (179), and remodeling of the actin cytoskeleton allows them to invade surrounding tissue and metastasize (145). Resistance to apoptosis allows the continued survival of the tumor in the face of DNA damage and removal from its normal cellular milieu (180). Resistance to oxidative damage allows cancerous cells to survive oxidative threats from the body's defense system and hypoxic conditions encountered in the interior of a tumor in the absence of vascularization (181). Many studies directly demonstrate or suggest that c-Src activation is able to induce all of these effects.

Src^{527F} expression results in activation of the MAPK pathway, which in turn induces cell cycle progression (182). It was also shown that Src^{527F} induces efficient rearrangement of the actin cytoskeleton, repositioning actin from stress fibers to actin based motility structures (183,184). c-Src activation also induces activation of the Akt/PI3K pathway, which in addition to its effects on the cytoskeleton, is an important mediator of cell survival and resistance to apoptosis (185). Finally, it was demonstrated that Src^{527F} induces high levels of HO-1 expression, a protein that is involved in the resistance to hypoxic stress (140).

The data of Summy et al. (191) indicated that chimeric constructs of Src^{527F} with the c-Yes amino terminus were only able to induce one of the effects mentioned above, at levels that approached those achieved by Src^{527F}: activation of the MAPK pathway. Unfortunately, due to the lack of a constitutively activated c-Yes, there is currently no data available in the literature on the phenotype of cells expressing this protein and how they differ from cells expressing active c-Src. $Y4U32^{527F}$ should functionally approximate constitutively active c-Yes (Yes^{535F}), as the only differences between the two proteins occur in their highly homologous tyrosine kinase domains. c-Yes is frequently activated in a subset of human cancers, most notably colon carcinoma and melanoma (141,164,169,171). In these cancers, the level of c-Yes activation correlates with the metastatic potential of the cells. How then does activated c-Yes contribute to the cancerous phenotype? Activation of the MAPK pathway may allow c-Yes to induce mitogenesis and thereby contribute to cell transformation. Tsygankova *et al.* demonstrated the importance of c-Yes in activation of the MAPK pathway in response to angiotensin II treatment of liver epithelial cells (63). Recent evidence also suggests that c-Yes may be important in regulation of cell adhesions (55) and VEGF-induced vascular permeability (92). c-Yes is most frequently activated in cancers of epithelial origin, thus c-Yes activation may contribute to metastasis by regulating tight junctions and/or through an increase in vascular permeability that would enhance angiogenesis.

While the data indicate that the c-Yes amino terminus prevents its participation in pathways that regulate actin filament rearrangement, it should be noted that palmitoylation of Src family kinases is a reversible process (99,101). As the SH4 domain is essential for the loss of function associated with the c-Yes amino terminus, regulated de-palmitoylation of c-Yes in cancerous cells may allow the protein to move out of lipid raft fractions and participate in signaling pathways that were previously inaccessible. As mentioned above, v-Yes is not palmitoylated and is capable of inducing PI3K activation, CEF transformation, and tumor formation in chickens (5,15,186). The palmitoylation status of activated c-Yes in cancerous cells has not been reported. Nevertheless, the results presented here indicate that the c-Yes amino terminus, including the SH4 palmitoylation signal and the Unique domain, are crucial for the inability of activated Src/Yes chimeras to induce actin filament rearrangement, activation of the PI3K/Akt pathway, and induction of HO-1 expression. The amino terminal region may thus limit the ability of c-Yes itself to contribute to the onset and progression of the metastatic phenotype. Thus, if activated c-Yes plays a role in cancer, we would predict that it would have to be displaced from interactions governed by the SH4/Unique domain before it could exert activation of PI3-kinase and changes in actin filament integrity that are a hallmark of transformation.

The experiments outlined in this review have examined the contributions of the non-catalytic functional domains to signaling specificity between the c-Src and c-Yes tyrosine kinases. The results indicate that each non-catalytic functional domain contributes to some aspect of signaling specificity. These results allow the proposal of a working model for specificity in signaling between c-Src and c-Yes (Figure 6). In this model, after synthesis, the two proteins are rapidly localized to cellular membranes. Upon membrane localization, c-Src and c-Yes differentially partition into detergent soluble and insoluble regions, respectively, as dictated by differences in their amino termini. This may be the most important contribution to signaling specificity between c-Src and c-Yes, as it determines the subset of proteins available for intermolecular interactions. Protein/protein interactions mediated by the Unique domain may contribute to membrane compartmentalization or stable complex formation with intermolecular binding partners that allow initiation of downstream signaling events. Protein/protein

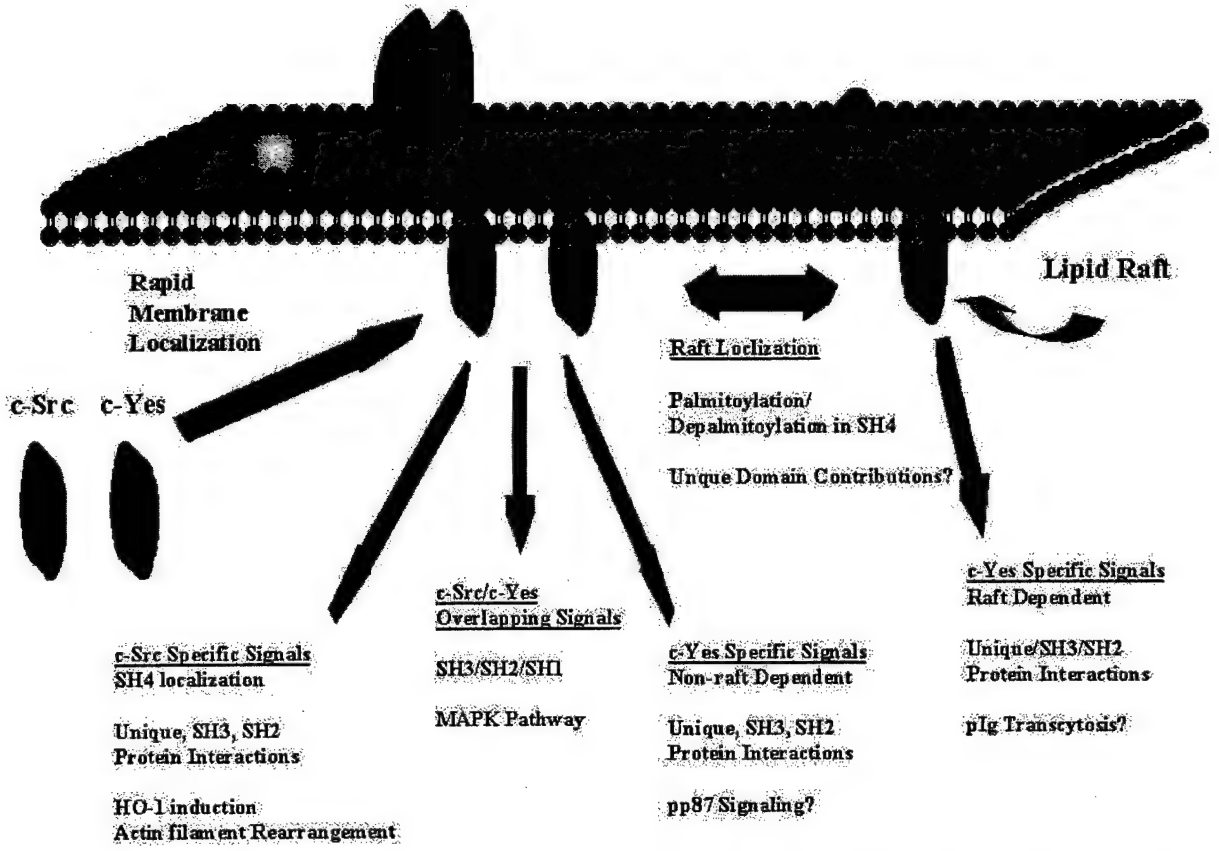

Figure 6. Model for Signaling Specificity between c-Src and c-Yes. In this model, after synthesis, c-Src and c-Yes are rapidly localized to cellular membranes. At the membrane, subtle differences in signaling between the two proteins may be mediated by differential protein/protein interactions through the SH3, SH2, and Unique domains. These protein/protein interactions may serve to affect subcellular localization, direct stable signaling complex formation, or select substrates for phosphorylation. The SH3, SH2, and SH1 (the tyrosine kinase domain) domains may also serve to transmit overlapping signals between the kinases. After membrane localization, c-Yes partitions into lipid raft fractions. The reversible nature of palmitoylation may allow c-Yes to traffic in and out of lipid rafts. Sequestration into lipid rafts may contribute significantly to c-Yes specific signaling by dictating the sub-set of proteins available for intermolecular interactions. The Unique domain may also contribute to localization. Once inside lipid rafts, c-Yes-specific signals may be dictated by protein/protein interactions mediated through the Unique, SH3, and SH2 domains.

interactions or phosphorylation events mediated by the Unique domains may also contribute to differential activation of c-Src and c-Yes, through destabilization of the closed conformation. Signaling specificity is further fine-tuned through intermolecular interactions mediated by the SH3 and SH2 domains. These regions serve to select substrates, and perhaps more importantly, direct stable associations with protein binding partners that may influence sub-cellular localization, activation state, and formation of multi-protein signaling complexes through which specific signals are sent. This model for signaling specificity does not exclude the ability of c-Src and c-Yes to function redundantly. Src family kinases are rapidly (5 min) localized to cellular membranes after synthesis, however, it was observed for Fyn that localization to detergent resistant lipid rafts occurs after a 10-20 min lag time (187), indicating potentially different mechanisms for membrane association. c-Yes and c-Src may thus interact with overlapping subsets of proteins before c-Yes partitions into lipid rafts. Additionally, regulated de-palmitoylation of c-Yes, and partitioning of other proteins between raft and non-raft fractions, may bring c-Src and c-Yes into contact with overlapping sets of proteins.

This model for signaling specificity between c-Src and c-Yes may be more broadly applicable to signaling specificity between Src family kinases in general. Understanding how these kinases are capable of sending specific signals will be important in the overall understanding of the function of these proteins, and in their possible usage as targets for rational drug design. Src family kinase inhibitors have shown promise as anti-cancer drugs due to their ability to block cell proliferation, however, most of these compounds do not distinguish

between Src family members (178,188,189). Studies in mice lacking one or more Src family members have revealed that loss of individual Src family kinases is more readily tolerated than loss of multiple Src family members (76). Thus, non-specific inhibition of Src family kinases may have deleterious effects as a result of disruption of multiple signaling pathways. A more ideal treatment strategy would involve inhibition of only the kinase or kinases that were abnormally activated in a particular tumor. A comprehensive knowledge of the roles of the functional domains in generating signaling specificity may allow the design of drugs specific for individual Src family kinases that would avoid the potentially deleterious effects of global Src family kinase inhibition.

10. SUMMARY

While the information available on c-Yes still lags behind that of c-Src and other members of the Src family, over the past decade, knowledge of the regulation and functions of the c-Yes tyrosine kinase have increased dramatically. It has become clear that c-Yes is capable of sending specific signals, however, the manner by which c-Yes accomplishes this remains a mystery. The works presented in this review have summarized c-Yes and the issue of signaling specificity between c-Src and c-Yes, with particular emphasis placed on the contributions of the functional domains to specificity in signaling between these closely related proteins. It is now clear that c-Src plays a key role in regulating a number of cellular signals associated with cell growth and changes in the cytoskeleton that are associated with transformation and cell motility. A function for c-Yes is less clear; however, several important observations point to a potential role in regulating cell-cell interactions and vesicle trafficking particularly in polarized cells. Each of the functional domains present in c-Yes appears to play some role in dictating specificity in signaling. A role for c-Yes in cancer is less clear, especially given the results of Summy et al., indicating that activated c-Yes does not direct changes in actin filaments that are normally associated with transformation (191). However, it is possible that distinct signals or changes that occur in cancer cells could direct activated c-Yes away from membrane microdomains and permit c-Yes to stimulate signaling cascades associated with transformation. With regards to cancer, the data of Summy et al., (191) indicate that activated c-Yes would be unable to induce activation of signals that alter actin filament integrity – a hallmark for transformation. This inability is based on the function of the SH4/Unique domain, which likely positions c-Yes in membranous regions of the cell that sequester it away from PI3-kinase, or permit it to interact with signaling proteins that preclude interactions with signaling partners that would direct activation of PI3-kinase and subsequent changes in actin filament integrity. Here, it was also noted that activate c-Yes is predicted to be unable to induce motility or invasion of chick embryo fibroblast cells. It was noteworthy that activated c-Yes was able to induce increased phosphorylation of Erk1/3, but unable to activate c-Raf. Thus, at first glance, it would appear that activated c-Yes would be unable to induce activation of cellular signals that direct transformation. However, in cancer cells, it is possible that activated c-Yes could be moved to different subcellular regions and stimulate transformation. This is evident based on that observation that chimeric constructs that replace either the SH4 region or Unique domain with the equivalent domains/regions of c-Src enable activated c-Yes to induce a transformed phenotype. Thus, activated c-Yes has the potential to induce transformation; however, we would hypothesize that SH4/Unique domain interactions prevent this.

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Research Paper

A Naturally Occurring Allele of BRCA1 Coding for a Temperature-Sensitive Mutant Protein

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ABSTRACT

Recent evidence suggests that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair. However, the mechanism of action of BRCA1 in either of these processes is still unknown. Here, we report the characterization of a disease-predisposing allele of BRCA1, identified in a family with several cases of ovarian cancer, coding for a protein that displays temperature-sensitive activity in transcriptional activation. The mutant protein differs from the wild type protein at a single amino acid, R1699W that occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologs. When the C-terminus of the mutant protein (aa 1560–1863) was fused to a heterologous GAL4 DNA-binding domain and expressed in yeast or mammalian cells, it was able to activate transcription of a reporter gene to levels observed for wild type BRCA1 at the permissive temperature (30°C) but exhibited significantly less transcription activity at the restrictive temperature (37°C or 39°C). Our results indicate that the transcriptional activity of the R1699W mutant can be modulated as a function of temperature and provide a novel experimental approach which can be utilized to dissect the molecular mechanism(s) of BRCA1 in processes related to transcription.

INTRODUCTION

Individuals carrying inactivating germline mutations in BRCA1 (OMIM 113705) have an increased predisposition to breast and ovarian cancer.[1,2] A growing body of evidence indicates that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair.[3-5] However, the mechanism of action of BRCA1 in either of these processes is still unknown.

During our analysis of BRCA1 germline missense mutations we came across a naturally occurring BRCA1 allele, identified in a family with several cases of ovarian cancer in which the mutation segregates with disease.[6] This allele carries a single point mutation (nucleotide C5214T), leading to a change from an arginine to a tryptophan residue at position 1699, located at the C-terminal region of BRCA1. The BRCA1 C-terminus (aa 1560–1863) has the ability to activate transcription when fused to a heterologous DNA binding domain (DBD) and introduction of disease-associated germ-line mutations impair transcription activation, while benign polymorphisms do not.[6-10] Missense mutations in BRCA1 that abolish transcription activation or disrupt the N-terminal RING finger structure also affect the ability of BRCA1 to interact with the RNA polymerase II holoenzyme in vitro and in vivo.[11,12] The C-terminal region encompasses two BRCT domains in tandem (BRCT-N [aa 1649–1736]; BRCT-C [aa 1756–1855])[13-15] and disruption of these domains is linked to cancer susceptibility. Interestingly, the ability to activate transcription does not seem to be a general characteristic of BRCT domains since BRCT domains isolated from other proteins, with the exception of RAP1, do not possess such activity.[16]

We previously observed that the R1699W mutant retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] Considering the conservation of basal transcription machinery in yeast and human cells,[17] we hypothesized that the discrepancy in transcription activity was due to differences at the temperature in which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. In the present study we demonstrate that the cancer-predisposing R1699W variant of BRCA1 acts as a temperature-sensitive mutant in both yeast and human cells in transcription activation assays.

MATERIALS AND METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα*, *ura3*, *trp1*, *his3*, 6 lexA operator-*LEU2*] contains a LexA-responsive *LEU2* gene, which when activated permits growth in the absence of leucine.[18] Transformations were performed using the yeast transformation system based on lithium acetate (Clontech) according to the manufacturer's instructions.

Yeast Expression Constructs. Constructs containing the fusion GAL4 DBD:BRCA1 wild type (amino acids 1560–1863) or mutants R1699W, M1775R and Y1853X were previously described.[6] BRCA1 inserts (wild type and mutants) were subcloned into pLex9 in-frame with the DBD of LexA. A *TRP1* selectable marker is present in pLex9, allowing growth in medium lacking tryptophan.

Yeast Growth Assay. Cells were transformed with the LexA DBD fusion constructs and plated in solid medium lacking tryptophan. At least three independent colonies for each construct were inoculated into liquid medium lacking tryptophan and grown to saturation (OD_{600} ~1.5) at 30°C. Saturated cultures were used to inoculate fresh medium lacking tryptophan or medium lacking tryptophan and leucine to an initial OD_{600} of 0.0002. Parallel cultures were then incubated at 30°C or 37°C and growth was assessed by measurement at OD_{600} after 38 hr.

Transcription Assay in Mammalian Cells. The region comprising the GAL4 DBD fused to BRCA1 C-terminus containing the R1699W mutation was excised from pGBT9 backbone[6] with *Hind*III and *Bam*H1 and ligated into pCDNA3. Constructs in pCDNA3 containing fusion of GAL4 DBD and wild type BRCA1, M1775R or Y1853X variants were previously described.[6] We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites[19] and transfections were normalized with an internal control, pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter using a dual luciferase system (Promega). Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in triplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C, 37°C or 39°C and harvested 16 hr later. Human cell lines NIH-OVCAR-3 and SKOV-3, both derived from ovarian adenocarcinoma, and CAOV-2, derived from the malignant ascites of a patient with progressive adenocarcinoma of the ovary, were kindly provided by Jeff Boyd (Memorial Sloan Kettering).

Western Blotting. Yeast cells were grown in selective media to saturation and OD_{600} was measured. Cells were harvested and lysed in cracking buffer (8M Urea; 5% SDS; 40 mM Tris-HCL [pH6.8]; 0.1 mM EDTA; 0.4 mg/ml bromophenol blue; use 100 ml per 7.5 total OD_{600}) containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a PVDF membrane. The blots were blocked overnight with 5% skim milk using TBS-tween, and incubated with the a-pLexA (for LexA constructs) monoclonal antibody (Clontech) using 0.5% BSA in TBS-tween. After four washes, the blot was incubated with the a-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-tween. The blots were developed using an enhanced chemiluminescent reagent (NEN, Boston, MA).

RESULTS

A Temperature-Sensitive Phenotype in Yeast. We previously observed that the R1699W mutant (Fig. 1) retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] This discrepancy was not due to differential protein stability, vector background or promoter stringency in the reporter.[6] To further investigate this phenomenon, we generated yeast cell lines with an inducible GAL4 DBD: R1699W BRCA1 fusion integrated in the yeast genome as a single copy. In this context, the R1699W variant also displays activity comparable to wild type BRCA1, ruling out the possibility that the results were due to abnormally high levels of the protein expressed by an episomal plasmid (results not shown).

We then reasoned that differences in transcription activation could be due to differences in the temperature at which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. To test this idea directly, we transformed *Saccharomyces cerevisiae* EGY48 with cDNAs coding for fusions of LexA (DBD) and the wild type C-terminal region of BRCA1 (aa 1560–1863) or constructs carrying either the R1699W mutant or one of two other germline disease-associated mutations, M1775R and Y1863X, as negative controls. Transcription activity was quantified at 30°C and 37°C using an integrated reporter gene (6 *lexA* binding sites; *LEU2*) that, when activated, allows growth in the absence of leucine. Cells carrying the wild-type BRCA1 construct were able to grow in selective medium lacking leucine at both temperatures (Fig. 2A). Conversely, cells carrying the two disease associated mutants did not show any detectable growth at either temperature (Fig. 2A). Interestingly, cells carrying the R1699W mutant were able to grow at levels comparable to the wild type at 30°C but growth was dramatically impaired at 37°C, indicating a marked reduction in transcriptional activity (Fig. 2A). Expression was comparable for the R1699W and the wild type protein at both temperatures (Fig. 2B).

The R1699W Variant Displays Temperature-Dependent Activity in Human Cells. To confirm the temperature-dependent activity of the R1699W variant, we cotransfected human kidney 293T cells with a luciferase reporter gene driven by GAL4-responsive promoter and cDNAs coding for fusions of GAL4 DBD with the wild type C-terminal region of BRCA1 (aa 1560-1863), the R1699W mutant or either of the two disease-associated mutations, M1775R and Y1863X, as negative controls. We then incubated cells in parallel at two temperatures: 30°C and 37°C. The disease-associated mutants M1775R and Y1853X display a small increase in relative activity at 30°C but their activity is significantly lower than the wild type BRCA1 (Fig. 3A, left panel). Interestingly, the transcriptional activity of the R1699W mutant was restored to wild type levels when cells were cultured at 30°C, indicating that this mutant acted as a temperature-sensitive allele of BRCA1 in transcription activation (Fig. 3A, left panel). Although at 37°C the R1699W mutant displays residual activity, experiments conducted at 39°C indicated a further reduction in activity (Fig. 3A, right panel). Expression was comparable for R1699W, M1775R and the wild type protein at both temperatures (not shown).

A Complex Regulation in Breast and Ovarian Cancer Cells. Considering the occurrence of multiple ovarian cancers (but not breast cancer) in the family in which the R1699W was identified, Lund 279, we next asked whether ovarian cancer cell lines were different from breast cancer cell lines with respect to the temperature-sensitive phenotype. We tested transcriptional activation in two

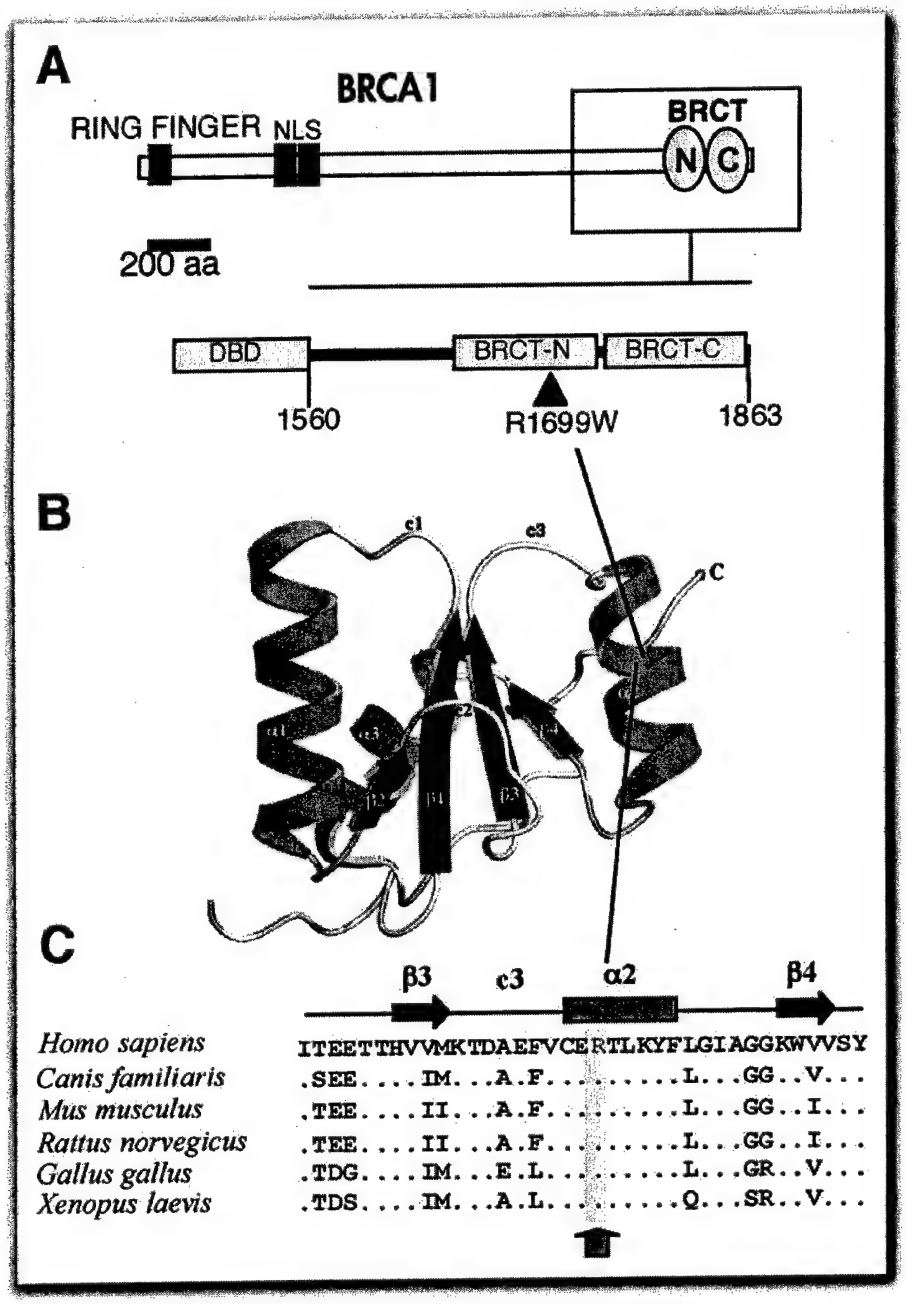

Figure 1. A temperature-sensitive BRCA1 mutant in transcription activation. (A) Top panel. Schematic representation of full length BRCA1 protein featuring: the RING domain in the N-terminus; the BRCT domains in the C-terminus (gray circles) and the nuclear localization signals (NLS). The region analyzed in this study is contained in the box, which is enlarged and represented in the bottom panel. Bottom panel. GAL4- and LexA-DNA binding domain (DBD) fusions to BRCA1 C-terminus (aa 1560–1863). The location of the R1699W mutation is indicated by a filled triangle. (B) Model of three-dimensional structure of BRCA1 BRCT (from ref. 30 by permission from Oxford University Press) indicating the location of the R1699 residue. (C) Alignment of BRCA1 homologs with secondary structures indicated on top. Dots represent identical amino acids. Location of R1699 residue is indicated with a red arrow.

breast cancer cell lines (MCF-7 and HCC1937) and three ovarian cancer cell lines (OVCAR-3, CaOV-2 and SKOV-3)(Fig. 3B). Surprisingly, the experiments revealed a complex regulation of this mutant in different human cancer cell lines. In MCF-7 and Caov-2, the R1699W allele was able to activate transcription to levels comparable to wild type BRCA1 at permissive and restrictive temperatures (Fig. 3B). In HCC1937 and NIH-OVCAR-3, the R1699W displayed temperature-dependent activity with normal activity at 30°C and loss of function at 37°C, consistent with our previous observation in 293T cells (Fig. 3A, B). Interestingly, in SKOV-3 cells the R1699W displayed a loss-of-function phenotype at both temperatures (Fig. 3B). In conclusion, our results did not reveal any correlation of the temperature-sensitive phenotype and tissue of origin and suggest that the R1699W variant may have a cell-type specific temperature-sensitive phenotype.

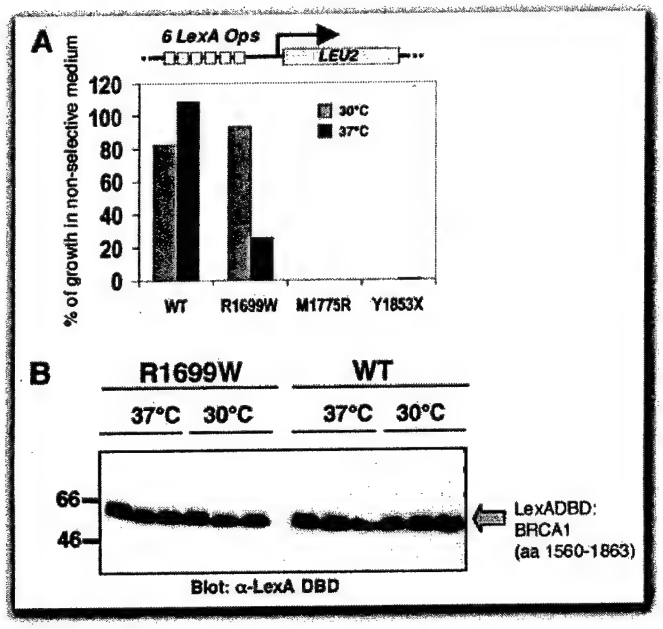

Figure 2. Transcriptional activity of BRCA1 R1699W at different temperatures in yeast. (A) Activity in yeast cells as measured by activation of an integrated LEU2 gene. Cells were cultured in non-selective and selective medium (lacking leucine) at 30°C and 37°C for 38 hr. Growth was measured by OD_{600}. Growth in non-selective medium was considered 100%. M1775R and Y1853X mutants were used as negative controls. (B) Mutant R1699W is expressed in yeast at the same level as wild type (gray arrow) at both temperatures. Three independent clones are shown for each condition. Blot was probed with α-LexA DBD monoclonal antibody.

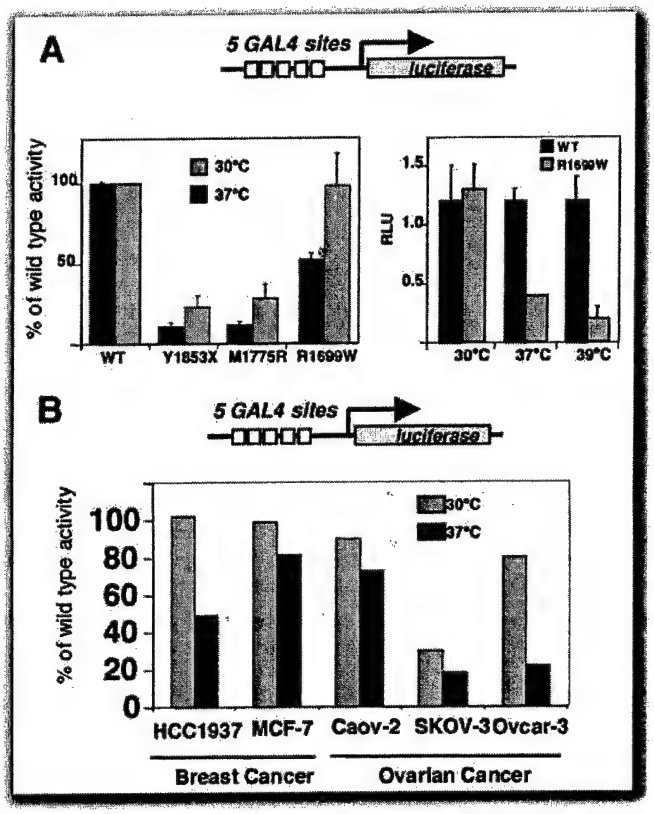

Figure 3. Transcriptional activity of BRCA1 R1699W at different temperatures in human cells. (A) Left panel. Transcriptional activity of the wild type BRCA1, R1699W, Y1853X and M1775R mutants at 30°C (gray bars) and 37°C (black bars). Activity of the R1699W variant in 293T cells is comparable to wild type at 30°C but markedly reduced at 37°C. Right panel. Transcriptional activity of the wild type BRCA1 and the R1699W mutant at different temperatures. RLU, relative luciferase units. Represents the ratio between Firefly luciferase and Renilla luciferase (internal control). (B) Temperature-sensitive activity of R1699W is cell type-specific. Average of three independent experiments. HCC1937, MCF-7 are breast cancer cell lines; CaOV-2, SKOV-3, NIH-OVCAR-3 are ovarian cancer cell lines. Structures of the reporters are depicted on top of the graphs. Transfections are normalized with a constitutive Renilla luciferase reporter.

DISCUSSION

The biochemical function of BRCA1 has remained elusive and the current evidence suggests that BRCA1 may have a pleiotropic function in the DNA damage response pathway and may be able to influence several activities that revolve around DNA damage resolution.[3,5,20-23] Alternatively, BRCA1 being a large multifunctional protein may have a wide range of unrelated biochemical activities in the cell. One approach to understand the function of a protein with tumor suppressor action is to analyze naturally occurring mutations that cause cancer predisposition.

In our study of missense mutations found in individuals with high risk for breast and ovarian cancer we came across a naturally occurring BRCA1 allele identified in a family from Lund that displayed unusual behavior in the transcription activation assay.[6] The clinical data suggests that the R1699W mutation (Arg to Trp substitution at codon 1699; see also Breast Cancer Information Core Database at http://research.nhgri.nih.gov/bic/) is likely to have a deleterious effect in vivo and predispose carriers to cancer. Disease association is further emphasized by the presence of the R1699W mutation in a large pedigree with several women diagnosed with ovarian cancer (Tom Frank, personal communication). Nonetheless, our initial transcription-based tests of this variant showed that it retained wild type activity in yeast but not in mammalian cells.[6] Previous studies had shown a complete concordance between results in yeast and mammalian cells.[7,8,24] This apparent divergence could not be explained by vector background, promoter stringency or abnormally high levels of the protein expressed by an episomal plasmid but rather was due to temperature differences at which the assays were carried out. Our results demonstrate that the R1699W variant display a temperature-sensitive phenotype in transcription activation and therefore may represent the first conditional mutant of BRCA1 to be described (Fig. 2).

The R1699W mutation is located in the BRCT domain, a region that is involved in binding to many different proteins that associate with BRCA1[3] and crucial for transcriptional activity.[10] The mutation, R1699W, replaces an arginine residue involved in a salt bridge that is thought to stabilize the packing of the two BRCT domains.[25] It occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologues (Fig. 1C). Interestingly, this region is not found in other BRCT motifs and seems to be unique to BRCA1 BRCT.[14,15] In fact, the predicted α-helix 2, in which the mutation resides, is conserved in all known BRCA1 homologs (Fig. 1C) and has been proposed to be responsible for determining functional specificity of the BRCT domains.[26]

Interestingly, Lund 279 presents almost exclusively ovarian cancer cases and no breast cancer cases raising the possibility of

differential effects of this mutant in breast versus ovary epithelia. That observation led us to test the temperature-sensitiveness in breast cancer and ovarian cancer cell lines. Intriguingly, our results indicate that the temperature-sensitive phenotype of the R1699W mutant is cell type specific but no tissue correlation was apparent with the cell lines tested (Fig. 3). At this point we can only speculate on the nature of such behavior and propose the following possible scenarios:

1. Residue 1699 is involved in binding to a factor required for transcription activation by BRCA1. The mutation would cause a marked decrease in binding affinity that is less severe at lower temperatures, therefore making it susceptible to variations in the concentration of this factor. For example, in cells where the factor is abundant, the R1699W variant would still be able to bind enough of the factor to promote transcription at both temperatures. Conversely, in cells in which the factor was at very low concentrations the R1699W variant would not be able to recruit the factor at either temperature. At intermediate concentrations of the factor, the R1699W variant would be able to bind it at 30°C but its ability to recruit the factor would be extremely reduced at 37°C.
2. Alternatively, it is also possible that instead of required for transcription, the function of this factor is to confer stability to the mutated protein (e.g., chaperone). In this case, the scenario of varying concentrations described above, or differential expression would also be applicable. This scenario is consistent with the observed increase in activity of the other mutants at 30°C (Fig. 2C).

Recently, mutants of the Xeroderma pigmentosum group D (XPD) helicase subunit of TFIIH displaying a temperature-sensitive phenotype in transcription and DNA repair have been isolated from patients with trichothiodystrophy (TTD). Patients carrying the mutant allele have a fever-dependent reversible deterioration of TTD features.[27,28] In this case, the phenotype manifests predominantly in the skin, hair and nails for reasons that are not well understood.[27] This finding raises important questions about the implications of the R1699W variant to the clinical phenotype. It is possible that small differences in temperature between breast and ovary may be responsible for an increased incidence of ovarian cancer. It remains to be seen if other families carrying this allele also display a preferential occurrence of ovarian cancer. These data also suggest that conditional mutants of BRCA1 in transcription may also have a conditional phenotype in the DNA damage response. We are currently exploring these possibilities.

In parallel with the present study we performed a random mutagenesis screen in yeast and identified 11 additional temperature-sensitive mutants of BRCA1.[29] Different from the variant described in this study, R1699W, which is an exposed surface residue, these additional TS mutants localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1.[29] Further characterization is needed to assess whether these conditional mutations of BRCA1 may serve as experimental tools to dissect the precise molecular role of BRCA1 in processes related to transcriptional regulation.

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Commentary

Participation of BRCA1 in the DNA Repair Response...Via Transcription

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Hereditary cancer syndromes, which usually represent a small portion of all cases, provide a genetically defined experimental system to understand the more common sporadic cases. For this reason the function of the breast and ovarian cancer susceptibility gene BRCA1 has been intensively pursued since its cloning and characterization in 1994.[1] Several lines of evidence indicate that its primary role is in the response to DNA damage but its precise biochemical function remains elusive (for a review see ref. 2).

BRCA1 has been implicated in a broad array of biochemical activities, some of them inferred by its association with other proteins of known function. These activities include transcriptional activation and repression, control of cell cycle checkpoints, participation in homology-directed recombination, transcription-coupled repair and ubiquitin ligase activity. Importantly, BRCA1 has been shown to be hyperphosphorylated in response to a wide variety of insults to DNA such as ionizing and UV radiation, hydroxyurea, hydrogen peroxide as well as several chemotherapeutic drugs. A conservative way of interpreting the current evidence is that BRCA1 is a link between the sensor and effector components of the response of DNA damage in mammalian cells. The finding that BRCA1 is recruited to sites of DNA damage earlier than Rad50 or Rad51 complexes suggest a role for BRCA1 in initial events following DNA damage.[3] One possible functional link is likely to be the regulation of transcription of genes encoding proteins that participate in the DNA damage response, including DNA repair. Consistent with this idea, the carboxy terminal domain of BRCA1 is capable of activating transcription and of interacting with the RNA polymerase II (for a review see ref. 4).

Early experiments demonstrated that BRCA1 cooperates with transcription factors such as p53 and STAT1 to induce the expression of the cell cycle inhibitor p21WAF1.[5-7] Array-based strategies later revealed that a major target for BRCA1 was the DNA damage responsive gene GADD45.[8,9] Interestingly, GADD45 was initially isolated as a gene induced by growth arrest and DNA damage agents.[10] Taken together these results suggested that BRCA1-mediated transcription would primarily guarantee a robust cell cycle arrest to allow the damaged cell enough time to repair its DNA.

Although there has been considerable interest in the response to ionizing radiation, only the initial events of BRCA1-mediated response to UV-induced damage have been studied. BRCA1 is phosphorylated after UV irradiation leading to relocalization of BRCA1 to PCNA+ complexes in S phase cells.[11] UV-induced phosphorylation is due primarily to the activity of the ATM and Rad3 related (ATR) kinase.[12,13] The downstream events and the biological implications of the ATR-mediated response are still unclear.

A new piece of the puzzle is provided by Takimoto et al[14] in this issue of Cancer Biology and Therapy. This study supports the role of BRCA1 in transcription activation and provides evidence that the transcriptional response to DNA damage mediated by BRCA1 is more pleiotropic than previously thought. In this study the authors show that BRCA1 collaborates with p53 to activate DDB2 following UV- and cisplatin-induced damage via a p53 responsive element present in the human DDB2 promoter. Although not essential for the p53-mediated transcription, the presence of BRCA1 enhances transcription activation of DDB2, the smaller subunit of the DDB heterodimer (composed of a 48 kDa and a 125 kDa protein). DDB binds to DNA damaged by UV or cisplatin and is mutated in a subset of patients with the cancer-prone syndrome xeroderma pigmentosum complementation group E.[15] Little is known about the function of DDB2 but recent evidence suggests a role in enhancing Global Genomic Repair of cyclobutane pyrimidine dimers.[16]

The study by Takimoto et al[14] also reinforces the connection between BRCA1 and p53. Besides the interaction of BRCA1 and p53 in transcriptional regulation, it has also been shown that inactivation of p53 partially rescues the embryonic lethal phenotype generated by disruption of Brca1 in the mouse.[17,18] Interestingly, inactivating mutations in p53 seem to be a hallmark of tumors arising in patients carrying germ-line mutations in BRCA1 or from Brca1-/-mice.[19,20] In addition, BRCA1 and p53 levels seem to be mutually modulated.[21,22] These results indicate that a significant part of the biological actions of BRCA1 involve p53.

Takimoto and colleagues show that the BRCA1-mediated induction of DDB2 gene expression following UV irradiation is ATM-independent, consistent with previous reports that the UV response is mediated primarily by ATR. It will be interesting to confirm this prediction as well as to dissect the roles of the various phosphorylation sites in BRCA1. By the same token, it will be important to determine whether the induction of DDB2 by BRCA1 following Adriamycin exposure (resulting in DNA double strand breaks) is dependent on ATM. It has been proposed that different types of DNA damage signal to BRCA1 in different ways and it is conceivable that targeting different phosphorylation sites might direct BRCA1 into pathways geared towards the resolution of a particular type of damage. In this scenario, the role of BRCA1 would be akin to a routing platform adapting specific responses to the immediate needs of the damaged cell.

It has become increasingly clear that cells lacking BRCA1 are more susceptible to DNA damage and might prove to be a particularly sensitive to current clinical regimens such as irradiation or chemotherapy agents that generate DNA damage. However, the eventual success of this approach may depend on an intact apoptotic response. If cells with extensive DNA damage in which the apoptotic program has been inactivated fail to be eliminated these treatments may in turn promote the development of the tumor. For these reasons, a further dissection of BRCA1 role in transcription and in the participation in the DNA damage response is warranted and may reveal clinical avenues for future management of breast and ovarian cancer.

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Research Article

Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription Activation

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KEY WORDS

BRCA1, Yeast, Yranscription, Temperature-sensitive mutants, BRCT domain

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ABSTRACT

BRCA1 is a tumor suppressor gene and germ line mutations account for the majority of familial cases of breast and ovarian cancer. There is mounting evidence that BRCA1 functions in DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular reagents to carry out biochemical and genetic experiments. Therefore, we used random mutagenesis of the C-terminus of BRCA1 (aa 1560–1863) to generate temperature-sensitive (TS) mutants in transcription activation. We obtained 11 TS mutants in transcription that localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1. One of the mutants, H1686Q, also displayed temperature-dependent transcription activation in human cells. These conditional mutants represent valuable tools to assess the role of BRCA1 in transcription activation.

INTRODUCTION

Germ-line mutations in BRCA1 confer high risk for breast and ovarian cancer.[1,2] The molecular function of BRCA1 is not yet known but there is increasing evidence that it is involved in DNA damage repair and gene transcription.[3,4] Several lines of evidence support a direct role for BRCA1 in transcription. When fused to a heterologous DNA binding domain (DBD) the C-terminus of BRCA1 activates transcription from a reporter gene and the introduction of cancer-associated mutations, but not benign polymorphisms, abolish[25] activation.[5-7] In addition, BRCA1 interacts with the RNA polymerase II and with several complexes involved in chromatin remodeling.[8-11] Ectopic expression of BRCA1 results in the transcription of genes involved in cell cycle control and DNA damage repair.[12-17] Interestingly, BRCA1 also interacts with CsTF50 in a complex that regulates mRNA processing pointing to a pleiotropic role in transcription.[18]

Despite the absence of BRCA1 homologs in its genome, yeast has been an important model system to study BRCA1 as well as the function of several mammalian transcription factors.[19] Yeast has been utilized to perform structure-function analysis of BRCA1 in transcription as well as to probe its mechanisms of activation based on the correlation with the clinical data.[5,7,20-22] In addition, overexpression of human BRCA1 in yeast generates a small colony phenotype that has been proposed as a method to classify uncharacterized mutations in BRCA1.[23,24] Thus, despite its limitations, yeast is a defined system to analyze BRCA1 function and is adequate for the rapid screening of large mutant libraries.

A major hurdle to define the function(s) of BRCA1 is the lack of molecular tools. Temperature-sensitive (TS) mutants would be particularly useful for this analysis. Recently, we have identified a BRCA1 allele in a family with familial ovarian cancer that displays a temperature-sensitive phenotype in mammalian cells (refs. 21, 41). Therefore, we hypothesized that a differential screen in yeast based on random mutagenesis would allow us to isolate additional TS mutants. We followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation and performed parallel screens at 30°C and 37°C.[20] We utilized this yeast-based system to identify and characterize 11 TS mutations and 15 loss-of-function (LF) mutants of BRCA1. One of the TS mutations identified in the yeast screen was found to exhibit a similar phenotype in human cells. These mutants will allow the study of BRCA1 function in yeast and provide a basis for the development of novel conditional mutants for mammalian cells.

METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3,* 6 *lexA* operator-*LEU2*] was co-transformed with the LexA fusion vectors and reporter plasmid pSH18–34, which has lacZ under the control of 8 LexA operators.[25,26] The LexA DBD fusion of wild type human BRCA1 C-terminus

(aa 1560–1863) and two germ-line mutants of BRCA1, Y1853X and M1775R were used as controls. Competent yeast cells were obtained using the yeast transformation system (Clontech).

Error-Prone PCR Mutagenesis and Screening. A 30-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using *Taq* polymerase, p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). PCR products were gel purified and co-transformed in an equimolar ratio with an *NcoI*-linearized wild-type pLex9 BRCA1 (aa 1560–1863) plasmid and pSH18-34. Transformants carrying the mutagenized cDNAs were plated at 37°C or 30°C on plates lacking tryptophan and uracil and containing 80 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 2% Galactose, 1% Raffinose, 1X BU salts (1L of 10X BU salts: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4). The X-gal plates allowed direct visualization and were scored after 6 days. Clones were recovered from yeast and sequenced.

Mammalian Cell Reagents. A region comprising the BRCA1 coding region containing the TS mutation in pLex9 vector was excised with *EcoR*1 and *Bam*H1 and subcloned in pGBT9 in frame with GAL4 DBD. The fusion GAL4 DBD: BRCA1 was then cut with *Hind*III and *Bam*H1 and ligated into pCDNA3. We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites and transfections were normalized using a dual luciferase system (Promega). For the mammalian two-hybrid system the pCDNA3 GAL4 DBD: BRCA1 (aa 1560–1863) and the constructs carrying different TS mutations were used as bait to test interaction against CtIP. The construct containing CtIP (aa 45–897) fused to the herpesvirus VP16 transactivation domain (aa 411–456) was used as target and the VP16 vector was used as negative control (gift from Richard Baer, Columbia University). Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in quadruplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C or 37°C and harvested 16 hr later.

RESULTS

Screen for TS Mutants of BRCA1 in Transcription Activation. We screened ~ 3 x 10^6 independent clones and recovered 1,302 putative LF mutants at 37°C (Fig. 1A). These colonies were then plated on fresh plates and incubated at 37°C and 30°C for confirmation (Fig. 1B). All plates contained yeast expressing wild-type cDNA to control for the different activity of β-galactosidase at both temperatures. Several clones turned out to display either a loss-of-function (white clones) or wild-type (blue clones) phenotype at both temperatures. Plasmids were recovered, retransformed into yeast and their activity confirmed. Clones that failed to display a reproducible activity were discarded. Plasmids representing 38 clones (3 were not recovered) were analyzed by restriction digest and although no clone had detectable deletions/insertions by gel analysis, sequencing revealed that 12 had nucleotide deletions or nonsense mutations and were not analyzed further. The remaining clones were processed for sequencing and the mutation identified. Eleven clones displayed markedly reduced activity at 37°C and wild-type activity at 30°C (TS clones; Table 1) and 15 had reduced activity at both temperatures (LF clones, Table 2).

TS Mutants in Yeast. Our screen resulted in the isolation of 11 TS mutants (8 unique) in transcription activation in yeast (Table 1). Seven clones displayed only one missense mutation and four clones displayed two missense mutations (Table 1). It is unclear whether the two mutations are required for the TS phenotype or not. At least in one case, TS32 (S1722F/K1667E), we know this is not the case because a similar mutation was found independently in another clone, TS25. Mutations causing TS activity were found in exons 16–20 and 24. Interestingly, conserved hydrophobic residues were found to be a major target of mutations followed by mutations in serine residues (Table 1 and Fig. 2). With three exceptions, S1631N, L1639S and E1836G, all mutations occurred either in the N-terminal BRCT region or in the interval between the N- and C-terminal BRCTs (Table 1 and Fig. 2).

Loss-of-Function (LF) Mutants. Due to the experimental design, several clones proved not to be TS mutants but instead LF mutants at both temperatures tested (Table 2). These mutations also targeted hydrophobic

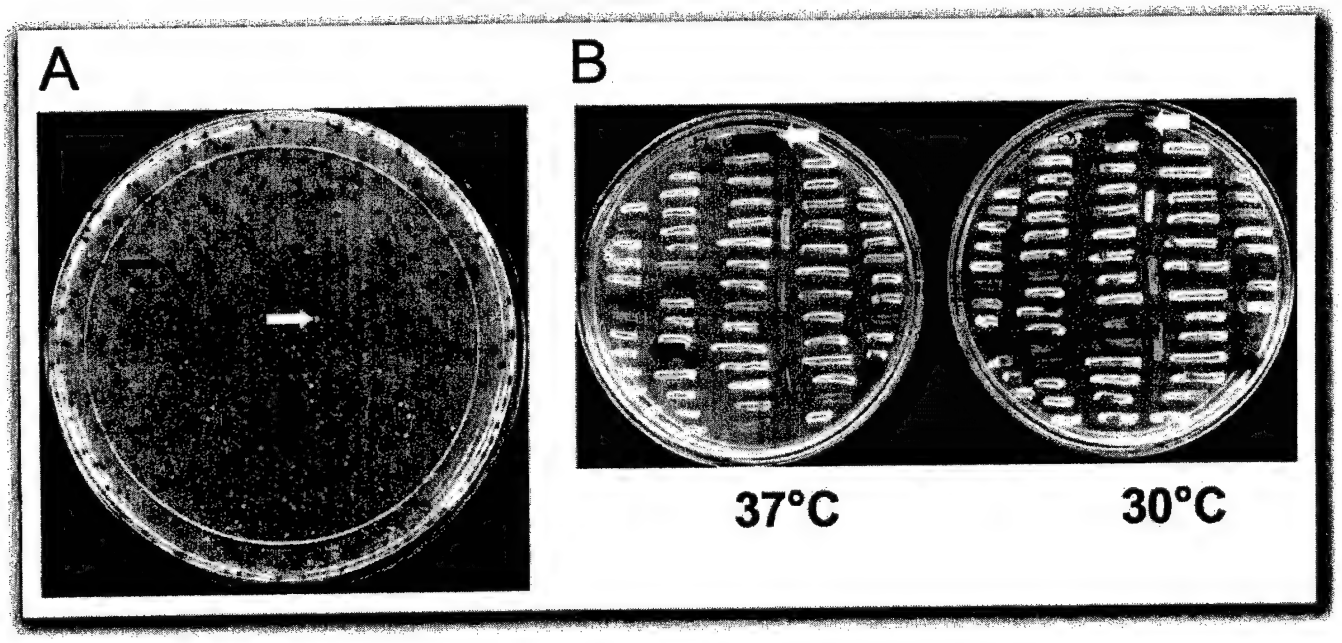

Figure 1. Screening for temperature-sensitive mutants of BRCA1 in transcription. (A) Primary screening at 37°C. Transformants carrying BRCA1 with wild-type activity appear as blue colonies (blue arrow) and transformants carrying loss-of-function mutants at 37°C appear as white colonies (white arrow). White colonies were replated in parallel and incubated at 30°C and 37°C. (B) Plates containing replicas of each white clone isolated from primary plates. A transformant carrying a wild-type BRCA1 is included at the top of each plate (white arrow). Clones that were consistently white at 37°C and blue at 30°C were isolated as temperature-sensitive mutants (blue arrow). Clones that were white at both temperatures were isolated as loss-of-function mutants.

Table 1 **TEMPERATURE-SENSITIVE MUTANTS OF BRCA1 (AA 1560–1863) IN TRANSCRIPTION**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	37°C
TS1	17	F1668S	T5122C	F	BRCT-N α-helix 1	+++	-
	24	E1836G	A5626G	DE	BRCT-C α-helix 3		
TS4	16	L1605L	T4932C	silent	unknown	+++	-
	17	V1687A	T5179C	V	BRCT-N β-sheet 3		
	19	K1727E	A5298G	KRQ	BRCT-N/BRCT-C interval		
TS6	16	L1639S	T5053C	LV	unknown	+++	-
TS19	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
	20	E1735E	A5324G	silent	BRCT-N/BRCT-C interval		
TS25	16	S1610S	T4949C	silent	unknown	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS26	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS30	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS32	17	K1667E	A5118G	KR	BRCT-N α-helix 1	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS33	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS36	16	S1631N	G5011A	SI	unknown	+++	-
	18	V1713A	T5257C	VI	BRCT-N β-sheet 4; uncharacterized variant found as a germline mutation[e]		
TS50	17	H1686Q	T5177A	H	BRCT-N β-sheet 3	+	-

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[37] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

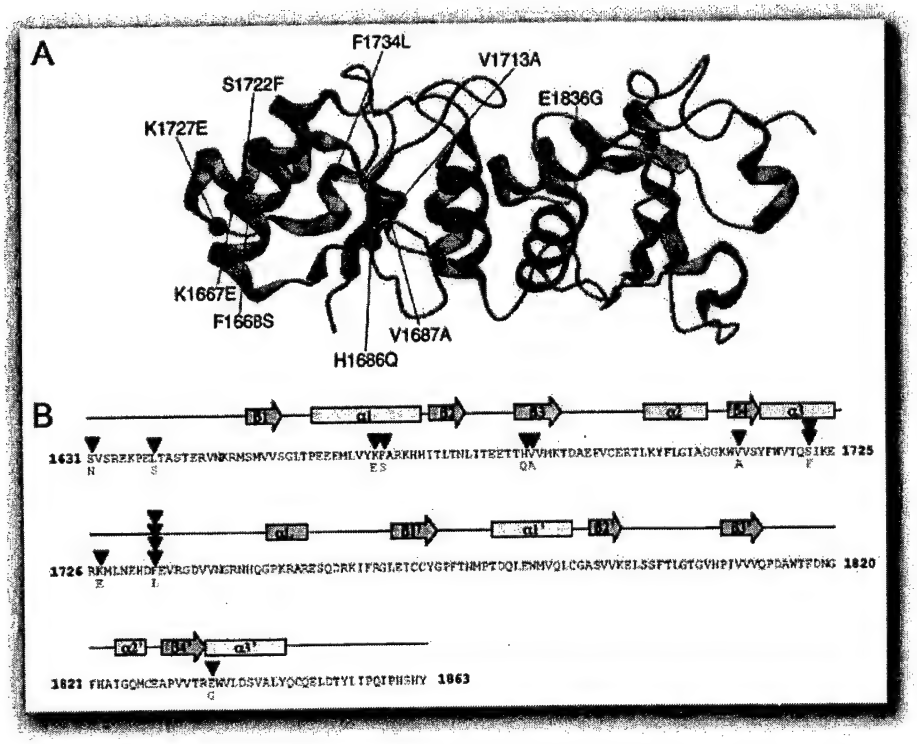

Figure 2. TS mutations localize primarily to the BRCT-N domain. (A) The location of the eight unique TS mutations is shown in the BRCT-N and BRCT-C domains of human BRCA1 according to the crystal structure of human BRCA1 BRCT region.[37] Red spheres represent the only coding change in a single clone and blue spheres represent changes that are in clones with multiple mutations. Note that with exception of S1631N, L1639S (which precede the BRCT domains and are not shown) and E1836G (TS1), all other TS mutations map to the BRCT-N domain. (B) Secondary structure elements according to crystal structure of BRCA1 BRCT region[37] are depicted above the sequence. Interval region, separating BRCT-N and BRCT-C is represented by a dotted line with a α-helix (αL; purple). Residue positions mutated in TS clones are shown for clones containing one (red triangle) or two changes (blue triangle). Changes are indicated below the sequence.

Table 2 **Loss-of-Function Mutants of BRCA1 (aa 1560-1863) in Transcription**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	Activity[d] 37°C
LF2	23	Q1811R	A5551G	Q	BRCT-C β3-α2 loop; uncharacterized variant found as a germline mutation[e]	-	-
	23	P1812S	C5553T	P	BRCT-C β3-α2 loop		
	24	A1843P	G5646C	AS	BRCT-C α-helix 3; uncharacterized variant found as a germline mutation[e]		
LF3	17	L1671L	A5132G	silent		-	-
	18	E1694G	A5201G	E	BRCT-N β3-α2 loop		
	24	V1842A	T5644C	VIL	BRCT-C α-helix 3		
LF5	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
LF8	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF15	24	A1843T	G5646A	AS	BRCT-C α-helix 3	-	-
LF20	17	T1691T	A5192G	silent	unknown	-	-
	17	F1668S	T5122C	F	BRCT-N α-helix 3		
	24	R1835R	A5624G	silent			
	24	P1856T	C5685A	PQS	unknown		
LF22	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF23	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF24	20	G1743R	G5346A	G	BRCT-N/BRCT-C interval	-	-
LF27	16	L1636L	T5034C	silent		-	-
	16	L1657P	T5089C	L	BRCT-N β1-α1 loop		
	17	L1664L	C5110T	silent			
LF28	16	S1577P	T4848C	S	unknown	-	-
	16	S1655P	T5082C	S	BRCT-N β1-α1 loop; residue mutated in the germline (S1655F)[e]		
LF35	21	M1775R	T5443A	M	BRCT-C β1-α1 loop; cancer-associated mutation found in the germline[e]	-	-
	22	Q1779Q	A5456G	silent			
LF34	23	I1807S	T5539G	IVL	BRCT-C β-sheet 3	-	-
	23	H1822H	T5585C	silent			
LF38	16	E1660G	G5098A	EKSC	BRCT-N α-helix 1	-	-
LF47	16	R1649R	A5066G	silent		-	-
	18	F1704S	T5230C	F	BRCT-N α-helix 2		

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[36] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

residues in the BRCT domains. Interestingly, we recovered a recurring cancer-associated mutation of BRCA1, M1775R (LF35; Table 2).[29] Also, Q1811R and A1843P, found together in LF2, are unclassified variants listed in the Breast Cancer Information Core database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively. Mutations causing LF phenotype were found in all exons examined with the exception of exon 19.

TS Mutants in Human Cells. All unique TS clones had their activity measured in human cells using a fusion to GAL4 DBD and a luciferase reporter driven by a GAL4-responsive promoter. Negative controls used were two cancer-associated mutants, M1775R and Y1853X.[29,30] In four independent experiments, one of the mutants (TS50) reproducibly displayed significant activity at the permissive temperature. Whereas at 30°C it exhibited approximately 30% of wild-type activity, at 37°C it did not activate transcription of the reporter (Fig. 3). Western blot analysis

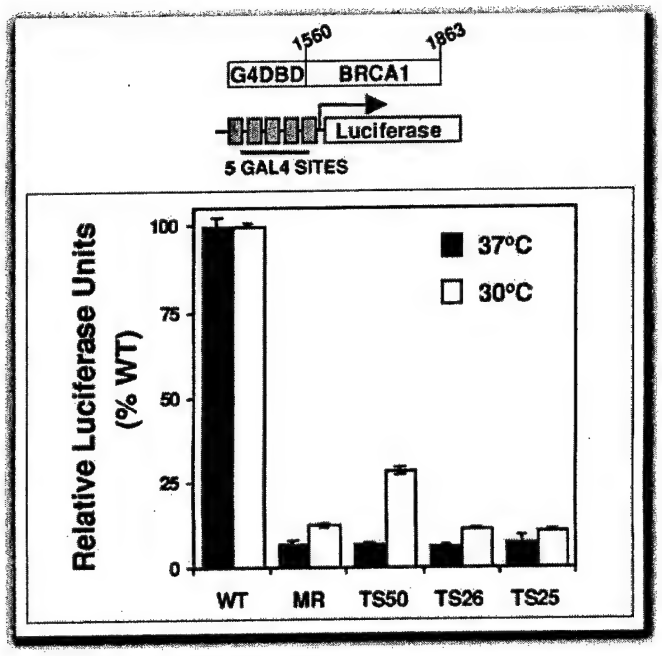

Figure 3. Transcriptional activity of TS mutants in mammalian cells. Upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein and of the luciferase reporter gene driven by five GAL4 binding sites. Lower panel depicts the activation of luciferase expression by wild type and mutant BRCA1 constructs in 293T cells at 37°C (solid bars) or 30°C (open bars). Data were normalized to the percentage of wild-type activity at each temperature. MR, BRCA1 (aa 1560–1863) carrying the cancer-associated M1775R mutation used as negative control.

revealed that all mutant constructs were being expressed, albeit at lower levels than the WT protein (not shown).

Mammalian Two-Hybrid System. Recent reports have demonstrated that CtIP, a protein involved in transcriptional repression and a substrate of ATM, interacts with the BRCT domains of BRCA1.[28,31-34] We next examined whether the TS mutants could interact with CtIP in a temperature-dependent manner in a mammalian two-hybrid assay. We reasoned that this assay would provide a complementary approach to assess the temperature-sensitive phenotype of the mutants. Our results confirm previous reports that CtIP interacts with the carboxy-terminal region of BRCA1 and show that this interaction also occurs at 30°C (Fig. 4).[28,31] Interestingly, TS26 and TS50 were found to interact with CtIP only at 30°C. The fold induction relative to the activity of the TS mutants transfected with the VP16 transactivation domain alone (7-fold and 10-fold, respectively) was less than fold induction obtained with the WT and CtIP:VP16, suggesting that the interaction at 30°C is only partially restored. Although we observed that TS26 interacts with CtIP, it failed to activate transcription at either temperature (Fig. 3).

DISCUSSION

The function of BRCA1 has remained elusive despite extensive effort to characterize its biochemical activities. It has been implicated in DNA repair, transcription activation and repression, transcription-coupled repair, mRNA processing, cell cycle checkpoint regulation and ubiquitination.[3,4,18,35,36] We reasoned that the isolation of conditional mutants would be an important addition in the experimental armamentarium to study BRCA1. Here we developed a screening strategy to isolate mutants of the BRCA1 C-terminus that display a TS phenotype.

Our screen isolated 11 unique loss-of-function (LF) mutants (Table 2), extending our analysis of mutants that affect transcription

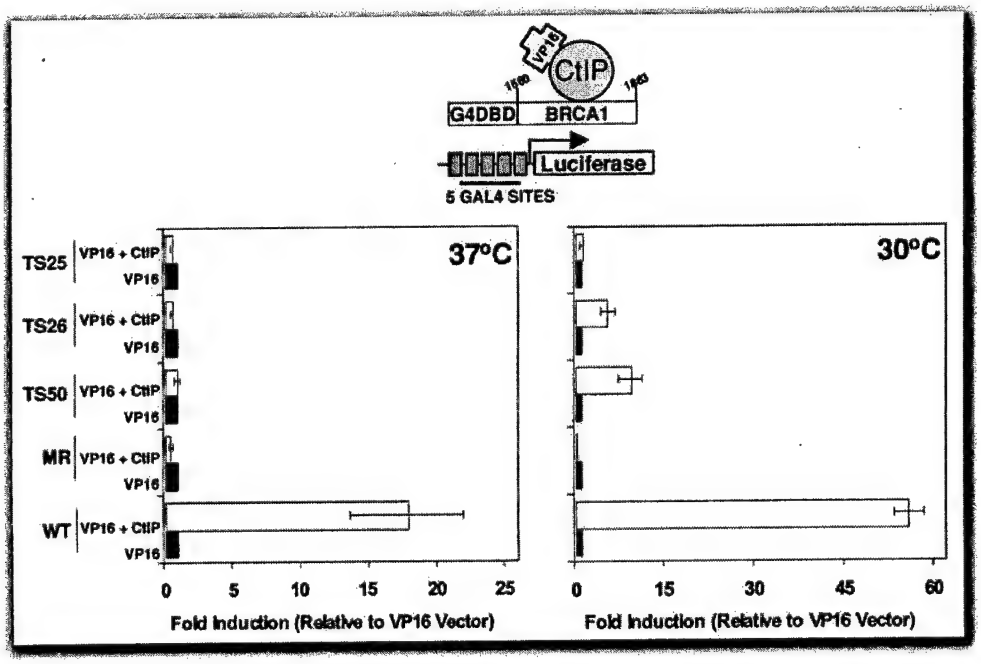

Figure 4. Mammalian two-hybrid system reveals a temperature-dependent interaction between BRCA1 TS mutants and CtIP. The upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein used as bait, the CtIP:VP16 fusion protein used as target and the luciferase reporter gene driven by five GAL4 binding sites. The lower panel depicts the activation of the reporter gene at 37° C or 30° C by wild type and mutant BRCA1 constructs in 293T cells cotransfected with empty VP16 vector (that codes for the VP16 transactivation domain alone) or vector containing CtIP (that codes for the CtIP:VP16 fusion protein). The data were normalized to show the fold induction of transcriptional activity for each TS mutant relative to its activity when transfected with VP16 vector alone.

activation by BRCA1 and allowing us to have a more detailed picture of the structure-function features of the C-terminal region of BRCA1.[20,21] The LF mutants recovered were localized primarily in conserved hydrophobic residues at the BRCT-N and the BRCT-C domains.

We have also isolated 8 unique TS mutants using the yeast screening (Table 1, Fig. 2). One mutation, F1734L, was found in 4 independent clones in our TS set and two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively, in the loss-of-function set. These findings suggest that the screen might have reached saturation and therefore the mutants recovered identify important regions for the regulation of BRCA1. To understand the functional consequences of these mutations we mapped the mutations onto the crystal structure of the BRCT domain region of BRCA1 (Fig. 2A).[27] Two mutations (S1631N and L1639S) mapped to regions outside the BRCT domain and were excluded from our analysis. Significantly, all other mutations leading to temperature sensitivity, with one exception (E1836G) mapped to secondary structures in the BRCT-N and to the interval region (Fig. 2B) and cluster preferentially at the hydrophobic core of the domain (Fig. 2A). The reason for this clustering is not known but it is possible that mutations the BRCT-C have more dramatic consequences for the general folding and therefore are not stable even at lower temperatures. Alternatively, the BRCT-N may provide an important binding site to the RNA polymerase II holoenzyme, an idea that is corroborated by in vitro binding studies of BRCA1 and RNA helicase A.[9] Therefore, for mutations in the BRCT-C to affect transcription their effect has to be more dramatic allowing us to isolate only loss of function mutations.

Interestingly, mutations in residues located at hydrophobic cores in the catalytic domain of tyrosine kinases as well as in SH3 domains have been demonstrated to confer temperature-sensitivity.[38] In three clones (TS1, TS4 and TS36) two mutations were found and only one of them may be important for temperature sensitivity. Alternatively, as found in TS mutants of v-Src, two mutations may be required.[39,40]

One of the TS mutants isolated in yeast, H1686Q, displayed a temperature-dependent activation of transcription when tested in human cells (Fig. 3). This observation indicates that residue H1686 is located at a critical position for the stability of the BRCT domains (Fig. 2). In addition to the ability of TS50 to activate transcription only at the permissive temperature in mammalian cells, we found that its interaction with CtIP also occurred in a temperature-dependent manner (Fig. 4). Intriguingly, mutant TS26 interacts with CtIP at the permissive temperature but is unable to activate transcription at either 30°C or 37°C. Based on these observations we propose that TS50 can be used to clarify the physiological relevance of the BRCA1/CtIP interaction.

The inability of most of these clones to behave as TS mutants in mammalian cells may be due to inherent differences in the range of temperatures and metabolism of yeast versus the mammalian system. Alternatively, this may reflect the fact that the reporter used in the screen is not stringent. We tend to favor the latter explanation because there are documented examples of TS mutants isolated in yeast screens at 25°C and 33°C, permissive and restrictive temperature respectively, that turned out to display TS activity in mammalian cells at 34°C and 40.5°C.[38] This is a striking example in which the permissive temperature in mammalian cells was even higher than the restrictive temperature in yeast suggesting that the mutants adapt to the range of temperatures used in a particular host. The use of a low-stringency reporter is important at the restrictive temperature to guarantee the selection of mutants with the lowest possible activity. However, when screened at the permissive temperatures it will allow the selection of clones that may have low activity. We are currently exploring these different possibilities.

Although only one of the mutant clones displayed a mammalian TS phenotype in transcription, the other clones isolated here are candidates to become molecular biological tools in yeast to dissect the function of BRCA1 in transcription and to guide further efforts to isolate more relevant TS mutants in mammalian cells. If we apply a conservative interpretation of the transcriptional assay, i.e., that it is a measure of the integrity of the BRCT domain, then it is possible that the data collected here may serve as a basis to rationally design conditional mutants to other proteins that present BRCT domains in their structure. It is important to stress that the TS mutants recovered are inactive at 37°C and are likely to represent cancer-associated variants if found as germ-line mutations.

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Functional analysis of *BRCA1* C-terminal missense mutations identified in breast and ovarian cancer families

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Germline mutations in the breast and ovarian cancer susceptibility gene *BRCA1* are responsible for the majority of cases involving hereditary breast and ovarian cancer. Whereas all truncating mutations are considered as functionally deleterious, most of the missense variants identified to date cannot be readily distinguished as either disease-associated mutations or benign polymorphisms. The C-terminal domain of *BRCA1* displays an intrinsic transactivation activity, and mutations linked to disease predisposition have been shown to confer loss of such activity in yeast and mammalian cells. In an attempt to clarify the functional importance of the *BRCA1* C-terminus as a transcription activator in cancer predisposition, we have characterized the effect of C-terminal germline variants identified in Scandinavian breast and ovarian cancer families. Missense variants A1669S, C1697R, R1699W, R1699Q, A1708E, S1715R and G1738E and a truncating mutation, W1837X, were characterized using yeast- and mammalian-based transcription assays. In addition, four additional missense variants (V1665M, D1692N, S1715N and D1733G) and one in-frame deletion (V1688del) were included in the study. Our findings demonstrate that transactivation activity may reflect a tumor-suppressing function of *BRCA1* and further support the role of *BRCA1* missense mutations in disease predisposition. We also report a discrepancy between results from yeast- and mammalian-based assays, indicating that it may not be possible to unambiguously characterize variants with the yeast assay alone. We show that transcription-based assays can aid in the characterization of deleterious mutations in the C-terminal part of *BRCA1* and may form the basis of a functional assay.

INTRODUCTION

Germline mutations in the breast and ovarian cancer susceptibility gene *BRCA1* (OMIM 113705) predispose carriers to early-onset breast and breast–ovarian cancer (1,2) and it is estimated that ~5% of all breast cancer cases are caused by inherited mutations in dominant disease genes. The majority of familial cases with both breast and ovarian cancer and a substantial part of families with breast cancer alone involve mutations in *BRCA1* (3). The *BRCA1* gene product is an 1863 amino acid phosphoprotein with a RING-finger motif at its N-terminus and two BRCA1 C-terminal (BRCT) domains at its C-terminus (1,4). With the exception of these domains, BRCA1 displays no similarity to other known proteins. The BRCT domains are mainly found in proteins involved in DNA repair, recombination and cell cycle control (5,6). Early findings suggest that BRCA1 is a tumor suppressor because loss of the wild-type allele was observed in familial breast and ovarian cancer cases (7). Although the function of BRCA1 remains unclear, there is increasing support for a role in DNA repair and transcription activation (for reviews see refs 8 and 9).

BRCA1 interacts with large protein complexes involved in DNA repair such as Rad51/BRCA2 (10,11) and Rad50/Mre11/p95 (12,13). Importantly, BRCA1 becomes hyperphosphorylated and disperses from Rad51-containing nuclear foci in response to DNA damage (14,15). In mice, Brca1 is required for transcription-coupled repair of oxidative DNA damage (16) and *Brca1*$^{-/-}$ embryonic cells accumulate genetic aberrations (17). However, no direct mechanism of action has been described which explains how BRCA1 exerts its functions.

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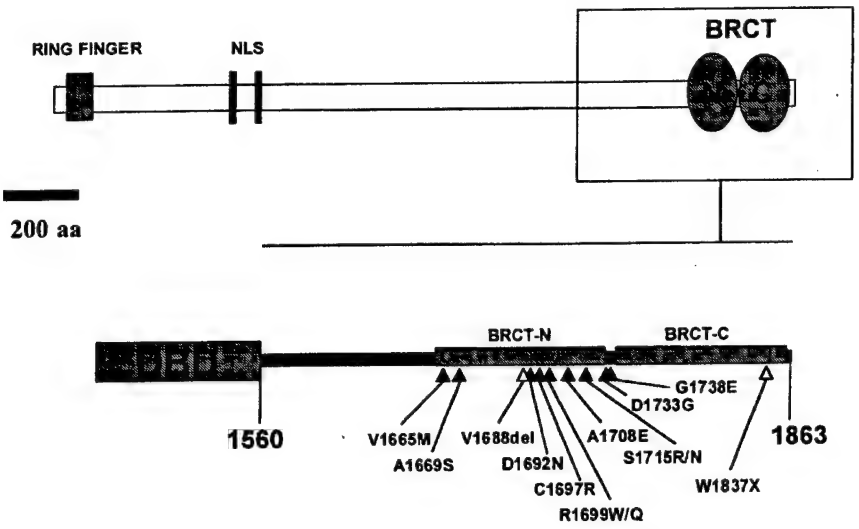

Figure 1. Domain structure of BRCA1. (Top) Schematic representation of full-length BRCA1 protein featuring the RING domain in the N-terminal region and the BRCT domains in the C-terminal region. The region analyzed in this study (amino acids 1560–1863) is contained in the box, which is enlarged and represented (bottom). Gray circles represent the two BRCT domains, BRCT-N (amino acids 1649–1736) and BRCT-C (amino acids 1756–1855). NLS, nuclear localization signals. (Bottom) GAL4- and LexA-DNA binding domain fusions to BRCA1 C-terminal region (amino acids 1560–1863). Mutations analyzed in this study are depicted as black (missense) or open (nonsense and in-frame deletion) triangles.

A transactivation activity was first ascribed to *BRCA1* by demonstrating that, when fused to a heterologous DNA-binding domain, the C-terminus of *BRCA1* acts as a transcription activator (18,19). BRCA1 associates *in vivo* with RNA polymerase II (pol II) holoenzyme as well as with the core pol II (20–22) and modulates transcription mediated by several transcription factors (9 and references therein).

The discovery of a transactivation activity revealed a testable function of BRCA1 and yeast-based assays have been proposed as a means of characterizing missense variants because disease-associated mutations abolish this activity (23,24). Numerous mutations in *BRCA1* have been described and established as disease-associated (Breast Cancer Information Core database, BIC). Such mutations are located throughout the gene and typically result in premature translation termination. Apart from a handful of clearly linked or strongly suspected disease-associated mutations, most amino acid substitutions reported hitherto cannot readily be distinguished as either disease-associated or benign polymorphisms and are classified as variants of uncertain significance (BIC), posing a very relevant problem in genetic counseling. Nevertheless, although the precise biochemical function of the protein remains unknown, increasing knowledge of the structural properties and biological roles of BRCA1 provides support in discriminating these alterations, eventually allowing functional assays to be developed (24,25). Yeast-based assays have been able to discriminate between disease-associated mutations and benign polymorphisms in the C-terminus of *BRCA1* (18,24,26,27). Therefore, it is tempting to suggest that the transactivation activity reflects a tumor-suppressing function of BRCA1 *in vivo*. Here we use a transcription activation assay to characterize the effect of unique germline variants identified in Scandinavian breast and ovarian cancer families. Seven of the included variants are of missense type and one is of nonsense type. In addition, we analyzed five C-terminal BRCA1 variants reported by others (BIC).

RESULTS

Analysis of hereditary breast and ovarian cancer has revealed several novel as well as previously described variants of *BRCA1*. Patients have been screened for mutations in *BRCA1* and *BRCA2* as described by Hakansson *et al.* (28). Here we analyze missense variants and one truncating mutation that localize to the C-terminal region of *BRCA1* (Fig. 1). These variants were not found in 50 healthy Swedish control individuals (no screen has been done for G1738E). Moreover, >450 index cases with familial history of breast–ovarian cancer have been screened for mutations in *BRCA1* and the variants reported here have been found only in their respective families, indicating that they represent rare variants.

We introduced the variants in constructs containing the fusion GAL4 DNA-binding domain (DBD):BRCA1 (amino acids 1560–1863) (Fig. 1) (18,26). In order to assess their transactivation activity these constructs were transformed into two *Saccharomyces cerevisiae* strains, HF7c and SFY526, containing reporter genes under the control of the GAL1 upstream activating sequence (UAS), recognized by GAL4 DBD. Wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and vector without insert was used as a negative control. Results were comparable in both yeast strains in a semi-quantitative assay (Table 1).

Table 1. Transcriptional activity of *BRCA1* variants identified in Scandinavian breast–ovarian cancer families and variants obtained from the BIC database

Family/ source	Exon	Mutation	Dog[a]	Mouse[b]	Rat[c]	Nucleotide[d]	Base change	Probable secondary structure elements[e]	Transcriptional activity		
									HF7c (His)[f]	SFY526 (β-gal)[g]	EGY48 (β-gal)[h]
Lund 321	17	A1669S	A	A	A	5124	G→T	α-helix 1 of BRCT-N	+	+	+
Lund 275	18	C1697R	C	C	C	5208	T→C	α-helix 2 of BRCT-N	–	–	–
Lund 279	18	R1699W	R	R	R	5214	C→T	α-helix 2 of BRCT-N	+	+	+
Lund 488	18	R1699Q	R	R	R	5215	G→A	α-helix 2 of BRCT-N	+	+[i]	+
Lund 20	18	A1708E	A	A	A	5242	C→A	α-helix 2/β-strand 4 loop of BRCT-N	–[j]	–[j]	Not done
Lund 184	18	S1715R	S	S	S	5262	A→C	β-strand 4/α-helix 3 loop of BRCT-N	–	–	–
Lund 32	20	G1738E	G	G	G	5332	G→A	BRCT-N/BRCT-C interval	–	–	–[k]
Lund 190	24	W1837X	W	W	W	5630	G→A	α-helix 3 of BRCT-C; conserved W in BRCT domains	–	–	–
BIC	17	V1665M	V	V	V	5112	G→A	α-helix 1 of BRCT-N	+	+	+
BIC	17	V1688del	I	I	I	5181	delGTT	β-strand 3 of BRCT-N	–	–	–
BIC	17	D1692N	D	D	D	5193	G→A	β-strand 3/α-helix 2 of BRCT-N	+	+	+
BIC	18	S1715N	S	S	S	5263	G→A	β-strand 4/α-helix 3 loop of BRCT-N	–	–	–
BIC	20	D1733G	D	E	E	5317	A→G	BRCT-N/BRCT-C interval	+	+	+[k]

[a]Amino acids correspond to predicted translation from canine *Brca1* cDNA (GenBank accession no. U50709).
[b]Amino acids correspond to predicted translation from murine *Brca1* cDNA (GenBank accession no. U68174).
[c]Amino acids correspond to predicted translation from rat *Brca1* cDNA (GenBank accession no. AF036760).
[d]Nucleotide numbering corresponds to human *BRCA1* cDNA (GenBank accession no. U14680). Alignment was performed using Vector NTI Multiple Sequence Alignment version 1.0.1.1.
[e]According to a BRCA1 BRCT model from Zhang *et al.* (29).
[f]Thirty-six individual colonies were streaked on solid SD medium lacking tryptophan and histidine and scored for growth after 2 days at 30°C. A positive score (+) was noted if growth was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if growth was visually identical to the negative control (vector with no insert).
[g]Thirty-six individual colonies were streaked on filter overlaid on solid SD medium and assayed for β-gal activity after 2 days at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative control (vector with no insert). Clones were scored 6 h after addition of X-gal.
[h]At least six individual colonies were streaked on filter overlaid on solid SD medium lacking tryptophan and uracyl and assayed for β-gal activity the next day at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative controls (M1775R and Y1853X). Clones were scored 2 h after addition of X-gal.
[i]Partly reduced β-gal activity.
[j]Published results, Monteiro *et al.* (18).
[k]Published results, Hayes *et al.* (24).

Analysis of variants identified in Lund families

Variants A1669S, R1699W and R1699Q displayed wild-type activity, suggesting that they represent benign polymorphisms (Table 1). For variant A1669S, the data from functional assays are in agreement with the pedigree analysis (Fig. 2). One of the affected family members did not carry the mutation and cases of uterine and very early-onset ovarian cancer indicate involvement of predisposing genes other than *BRCA1* or *BRCA2*. Additional clinical data should provide insight regarding A1669S and will serve as measurement of the prediction provided by the assay. Interestingly, pedigree analysis seemed to indicate that the R1699W is a cancer-predisposing allele (Fig. 2, Lund 279). Disease association is further emphasized by other findings where R1699W was found in a large pedigree in which several women, through three generations and across four degrees of relatedness, diagnosed with ovarian cancer carried the variant (T.S. Frank and J. Scalia, in preparation). Disease association is less clear for R1699Q, found in a patient diagnosed with breast cancer at the age of 39 but without familial history of disease. Others found this variant in an unaffected individual, whose mother was diagnosed with premenopausal breast cancer and considered to be an obligate carrier of the R1699Q variant and whose grandmother was diagnosed with ovarian cancer at the age of ~60 years but without known mutation status (T.S. Frank, personal communication). The apparent discrepancy between the family and functional data prompted further examination of the R1699 variants.

Variant W1837X results in a truncated protein lacking the last 27 residues and displayed loss of activity. Smaller truncations (11 residues) have been linked to disease (2) and shown to confer loss of function in transcription and in small colony phenotype assays (18,27). Our result is in agreement with pedigree analysis in which the mutation segregates with the disease (Fig. 2, Lund 190).

Variants C1697R, A1708E, S1715R and G1738E displayed loss of activity, suggesting that they represent disease-associated

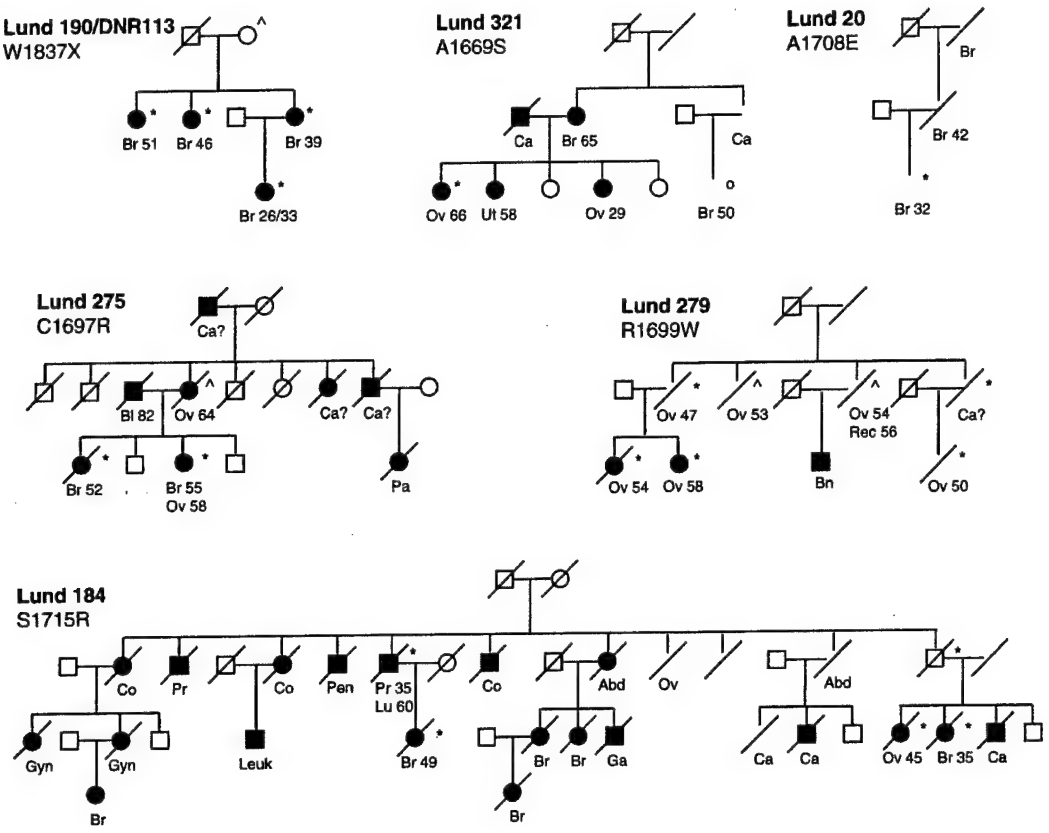

Figure 2. Scandinavian breast and breast–ovarian cancer families with germline *BRCA1* C-terminal missense or truncating mutations. Cancer types, age at diagnosis and mutation status are shown. *, mutation; °, confirmed from blood sample not to carry mutation; ^, determined from paraffin embedded tumor tissue not to carry mutation. Cancer type: Br, breast; Ov, ovary; Ut, uterus; Gyn, gynecological; Pr, prostate; Co, colon; Rec, rectal; Leuk, leukemia; Lu, lung; Bn, brain; Bl, bladder; Pa, pancreas; Ga, gastric; Pen, Penile; Abd, abdominal; Ca, cancer of unknown type; Ca?, possibly affected.

mutations (Table 1), an observation in agreement with pedigree analysis for mutations C1697R, A1708E and S1715R (Fig. 2). The amino acid substitution C1697R is a rather dramatic one, from a non-polar residue capable of forming disulfide linkages to a positively charged residue, located in a critical α-helix based on the structure of XRCC1 BRCT (29). Furthermore, the residue in question is strictly conserved in other *BRCA1* homologs (Table 1) (30,31). In addition to family Lund 275, in which it segregates with the disease, the C1697R variant has been found in three other breast cancer patients. One case had multicentric disease at age 35 and a family history of breast cancer (sister and mother), whereas the other two cases had bilateral disease at ages 41 and 44 and mothers with breast/skin cancer and cancer of unknown origin, respectively (J.T. Bergthorsson *et al.*, unpublished data). Thus, combined clinical data indicate association between the variant and breast cancer. Variant A1708E has been reported to the BIC database 14 times, including our finding in Lund 20. It has been previously shown to cause loss of function in different assays (18,19,27) and the presence of A1708E in Lund 20 further demonstrates the variant as a disease-associated mutation. S1715 is an evolutionarily conserved residue. However, the disease pattern in Lund 184 (harboring an S1715R substitution; variant S1715N was also analyzed) is not satisfactorily explained by a mutation in *BRCA1* alone because it presents an uncharacteristic phenotype. Multiple cases of colon cancer might suggest the involvement of a mismatch repair gene defect. However, co-segregation between the mutation and breast and ovarian cancer is observed and these cancer forms are predominant among women in the pedigree (Fig. 2). We recently found the G1738E variant, which displayed loss of transactivation activity in our assays, in a young patient affected with bilateral breast cancer and a family history of disease. In addition, others found the variant in a family with a strong pattern of hereditary disease in which the patient carrying the alteration suffered from breast cancer at an early age (T.S. Frank, personal communication). These findings strengthen the correlation between disease predisposition and predictions made by the transcription assay (24).

Analysis of variants in the BIC database

Variants V1665M, D1692N and D1733G displayed wild-type activity, suggesting that they represent benign polymorphisms

(Table 1). The V1665M variant affects a residue close to A1669, in the predicted BRCT conformation (29), which also displayed wild-type activity (Table 1 and Fig. 3), suggesting that this small stretch is tolerant to mutations. Variant D1692N affects the residue predicted to form a salt bridge with S1715, thereby stabilizing the interactions between BRCT α2 and α4 regions (29). However, D1692N displayed wild-type transactivation activity, suggesting that the predicted salt bridge is not important for the transactivation ability of BRCA1 (Table 1 and Fig. 3). The D1733G variant is a conserved acidic residue located in the BRCT-N/BRCT-C interval. However, more information is still needed for a reliable characterization of this variant.

Variants V1688del and S1715N displayed loss of activity, suggesting that they represent cancer-associated mutations (Table 1). Alteration V1688del is an in-frame deletion of a conserved hydrophobic residue predicted to be part of β3 in the BRCT-N domain (29). Previous mutation analysis has underscored the importance of hydrophobic residues for the function of BRCA1 (24). Similar to S1715R (Lund 184), the substitution S1715N (BIC) resulted in loss of activity in the assay.

Fusion protein and promoter stringency do not influence assay outcome

To rule out the possibility that the results obtained with the GAL4 DBD fusions were dependent on the DBD, we also performed the experiments using fusions to LexA DBD in the *S.cerevisiae* strain EGY48 (24). A fusion of wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and two mutants defined by genetic linkage as disease-associated, M1775R and Y1853X, were used as negative controls (1,2). Results from the LexA-based and GAL4-based assays were comparable (Table 1).

The reporter genes used in the yeast experiments contain multiple binding sites in their promoters (eight for LexA; four for GAL4), raising the possibility that variants with partial loss of function could score as wild-type in the semi-quantitative filter β-galactosidase assay. This could be particularly important in the case of the R1699W variant for which we found a contradiction between the family data and transcription activity. Therefore, EGY48 experiments with the R1699W variant were performed with the *LacZ* reporter under the control of one, two or eight LexA operators (32). In all cases, R1699W was indistinguishable from the wild-type allele (data not shown).

Quantitative assessment of transcription activation

Despite the fact that we saw no difference that could be attributed to promoter stringency, it was still possible that variants with partial loss of activity could be differentiated only using quantitative liquid β-galactosidase assay. However, results were comparable to the semi-quantitative assays (Fig. 3A and Table 1). Interestingly, R1699W was ~2-fold more active than the wild-type control. In conclusion, the contradiction found for variant R1699W was not due to a partial loss of function indistinguishable from the wild-type in semi-quantitative assays.

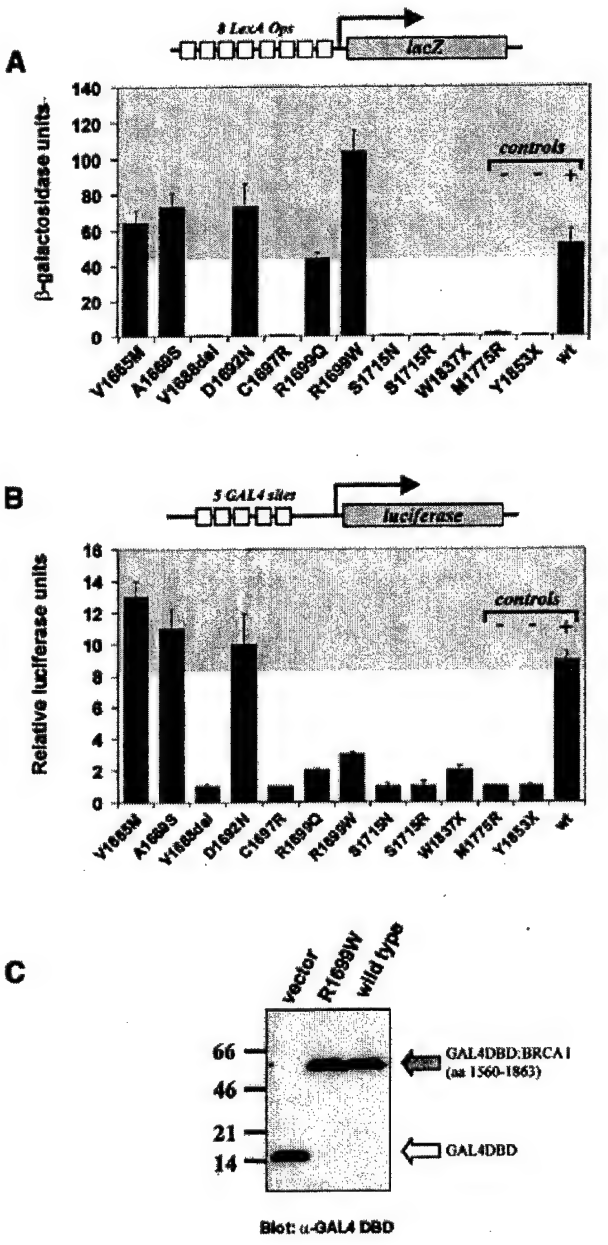

Figure 3. Transcriptional activity of BRCA1 variants. (**A**) Activity in yeast cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (**B**) Activity in human cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (**C**) Mutant R1699W is expressed at the same level as wild-type (gray arrow). The white arrow indicates expression of the GAL4 DBD moiety in the absence of any fusion fragment.

Analysis in mammalian cells

To further examine the transcription activity of the variants we performed assays in mammalian cells. With the exception of variants R1699W and R1699Q, transcription activation was comparable between yeast and mammalian cells (Fig. 3A and B). In 293T cells, variants R1699W and R1699Q displayed loss of function phenotype in accordance with pedigree analysis, suggesting that these variants are indeed cancer-associated mutations. Protein levels of R1699W and wild-type were similar, ruling out increased instability of the protein as the cause for the loss-of-function phenotype (Fig. 3C).

DISCUSSION

The notion that cancer-predisposing mutations in tumor-suppressor genes cause a loss-of-function phenotype is a key concept in cancer genetics. Here we utilized a functional assay to characterize clinically relevant *BRCA1* variants. Our rationale was that transactivation activity of BRCA1 might mirror a functionally important feature of the protein *in vivo* and form the basis for a functional assay. Several lines of evidence have called attention to BRCA1 as a transcription regulator and it has been demonstrated that disease-associated mutations abolish the transactivation by BRCA1 in different experimental systems (for a review see ref. 9). Importantly, BRCA1 alleles carrying benign polymorphisms retain wild-type activity (24,26). Thus, relevant functional information might be gained from characterizing the effect of *BRCA1* mutations on transcription activation. In addition, development of a functional assay for BRCA1 will fill a gap within the field directed at providing risk assessment information for counseling. The main difference between the present and past studies (24,25) is that this study is combined with pedigree and segregation analysis, providing a background to validate the results.

As demonstrated by our results in Table 1, the effect of an introduced *BRCA1* mutation on transcription activation in the yeast-based assay is not affected by the DBD of the fusion protein or the promoter context of the reporter gene. Problems in interpreting results might nevertheless arise when characterizing variants that do not affect protein function in yeast. This is exemplified by the R1699 variants (Table 1). Although the clinical data indicate that R1699W is likely to predispose carriers to ovarian cancer (Fig. 2), our yeast-based tests revealed a wild-type activity, an apparent divergence between disease predisposition *in vivo* and the transcription activation assay (Table 1). This disagreement could not be explained by vector background or by differences in promoter stringency. However, we found that in the mammalian cell-based assay, transactivation activity of the R1699 variants was reduced in a fashion comparable to the negative controls. In fact, all variants presented here, with the exception of R1699W and R1699Q, behave similarly in the yeast- and mammalian-based assays. Thus, it is possible that specific protein alterations that have an effect on *in vivo* phenotype remain undetected in the simplified yeast model. We are currently investigating the reasons for this difference. Consequently, at this time we cannot unambiguously characterize variants that do not disrupt transcription activation in yeast as benign polymorphisms. Using a mammalian-based assay to supplement results from the yeast assay might provide the scrutiny necessary to exclude or confirm disease predisposition of a certain variant. Similarly, mutations that affect mRNA processing *in vivo* might also be erroneously scored as a benign polymorphism because our assay is based on expression from an artificial cDNA. This could be the case for variant D1692N because the alteration affects a conserved guanine at a splice donor site and its potential effects on mRNA have not been examined. Conceivably, false negative results (i.e. benign polymorphisms that behave as loss-of-function mutants) can also occur when a particular variant causes message or protein instability in yeast. By extending our analysis using mammalian cells we should be able to distinguish those variants.

Considering the excellent correspondence between genetic alterations associated with breast and ovarian cancer in families and those that abolish transactivation, we tentatively characterized several additional *BRCA1* unclassified variants. We propose that variants V1665M, D1692N and D1733G represent benign polymorphisms and variants V1668del and S1715N represent disease-associated mutations. Final characterization of these variants must await independent confirmation.

Our findings, taken together with previously published data (18,24,26), demonstrate a correlation between loss of transactivation activity and disease predisposition and it will be interesting to see whether future data will corroborate the predictions made here. Our results indicate that yeast-based assays can aid in the characterization of deleterious mutations in the C-terminal part of *BRCA1* but it may be unable to unambiguously characterize benign polymorphisms. This is exemplified by mutations at residue R1699, for which we report a discrepancy in effect on transcription between yeast and mammalian cells. Thus, our study underlines the importance of analyzing the effect of putative disease-causing mutations in mammalian-based assays and taking into account data from population-based studies. In summary, we show that transcription activation may reflect the tumor-suppressing function of BRCA1 and provide further support for the role of missense mutations in disease predisposition.

MATERIALS AND METHODS

Yeast strains

Three *S.cerevisiae* strains were used: HF7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)3-CYC1-lacZ) (33); SFY526 (MATa, ura3-52, his 3-200, ade 2-101, lys 2-801, trp 1-901, leu 2-3, 112, canr, gal4-542, gal80-538, URA3::GAL1-lacZ (34); and EGY48 (MATa, ura3, trp1, his3, 6 lexA operator-LEU2) (35). HF7c and SFY526 contain reporter genes under the control of *GAL1* UAS, which is recognized by GAL4 DBD. When activated, the reporter gene in SFY526 will produce β-galactosidase and HF7c will grow in minimal medium lacking histidine. EGY48 cells were transformed with plasmid reporters under control of LexA operators (pSH18-34, pJK103 or pRB1840) that produce β-galactosidase when activated (35).

Yeast expression constructs

A fusion construct containing GAL4 DBD:BRCA1 (amino acids 1560–1863) in pGBT9 (Clontech) used as a wild-type control and as a backbone to introduce mutations was described by Monteiro *et al.* (18). Specific alterations in *BRCA1* were introduced by Quick-change site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. In short, primers containing the alteration were used in a PCR reaction to copy wild-type constructs produced in a methylation-competent bacterial strain and amplification was performed using *Pfu* polymerase. *Dpn*I was subsequently added to digest the parental plasmid, leaving only cDNAs with introduced mutations to be transformed into bacteria. Confirmation of the introduced mutations was obtained by direct sequencing of the *BRCA1* (amino acids 1560–1863) insert using two primers: GAL4 DNA-BD, 5′-TCATCGGAA-GAGAGTAG-3′ (17-mer) (Clontech), and pGBT9 M13 REV, 5′-TGTAAAACGACGGCCCGTTTTAAAACCTAAGAGT-CAC-3′. For experiments in EGY48, BRCA1 inserts with mutations were subcloned into pLex9 (35) in-frame with the DBD of LexA. Both pGBT9 and pLex9 have *TRP1* as a selectable marker, allowing growth in medium lacking tryptophan.

Yeast transformation

Transformations were performed using the yeast transformation system based on lithium acetate (Clontech). Briefly, a single colony was inoculated in YPD medium for 16–18 h to produce a saturated culture. Cells were transferred to fresh medium and grown for 3 h, centrifuged, washed, resuspended in TE/LiAc (10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution and used immediately for transformation. Competent cells were incubated in polyethylene glycol (PEG)/LiAc (40% PEG 4000, 10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution at 30°C for 30 min with appropriate vector and carrier DNA. DMSO was added to 10% final concentration and the mix was heat shocked at 42°C for 15 min. Cells were subsequently chilled, centrifuged and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Cells were plated on synthetic dropout medium (SDM) and incubated at 30°C to select for transformants.

Yeast growth assay

Thirty-six individual HF7c clones for each variant were streaked on solid SDM lacking tryptophan and on SDM lacking both tryptophan and histidine and growth was scored after 2 days. A positive (+) or a negative (–) score was noted if growth was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively.

β-galactosidase assays

Thirty-six individual SFY526 clones and at least six individual EGY48 clones for every variant were streaked on filter paper overlaid on solid SDM lacking tryptophan (or tryptophan and uracil for EGY48). Plates were incubated for 2 days (SFY526) or 24 h (EGY48) and cells growing on the filter paper were lysed by freeze–thawing in liquid nitrogen and assayed for β-galactosidase activity in 2.5 ml of Z buffer (16 g/l $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4{\cdot}7H_2O$, pH 7.0) containing 40 µl of X-gal solution (20 mg/ml in *N,N*-dimethylformamide) and 6.6 µl of β-mercapto-ethanol. For SFY526, a positive (+) or a negative (–) score was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively. For EGY48 a positive score (+) was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was identical to the negative controls (M1775R and Y1853X). Clones were scored 6 h (SFY526) or 2 h (EGY48) after addition of X-gal. Liquid assays were performed as described by Brent and Ptashne (36). At least three separate transformants were assayed and each was performed in triplicate.

Transcription assay in mammalian cells

GAL4 DBD:BRCA1 fusions were subcloned into pCDNA3 (Invitrogen). We used pG5Luc, which contains a firefly luciferase gene under the control of five GAL4 binding sites (37). Transfections were normalized with an internal control, pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter using a dual luciferase system (Promega). Human 293T cells were cultured in DMEM supplemented with 10% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were performed in triplicates using Fugene 6 (Roche) and harvested 24 h post-transfection.

Western blot

Cells were lysed in RIPA (150 mM NaCl, 10 mM Tris–Cl pH 7.4, 5mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% sodium deoxycholate), boiled in sample buffer and separated on a 10% SDS–PAGE. Gels were electroblotted on a wet apparatus to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked overnight with 5% skim milk using TBS–Tween, and incubated with α-GAL4 DBD monoclonal antibody (Clontech) using 0.5% bovine serum albumin in TBS–Tween. The blots were subsequently incubated with the α-mouse IgG–horseradish peroxidase conjugate in 1% skim milk in TBS–Tween and developed using an enhanced chemiluminescent reagent (NEN).

Electronic database information

Online Mendelian Inheritance in Man (OMIM) is available at http://www.ncbi.nlm.nih.gov/omim. The Breast Cancer Information Core (BIC) is an online database of mutations in breast cancer susceptibility genes hosted by the National Human Genome Research Institute and can be accessed at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

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BRCA1: exploring the links to transcription

Alvaro N.A. Monteiro

Progress on determining the function of the breast and ovarian cancer susceptibility gene *BRCA1* suggests it might be involved in two fundamental cellular processes: DNA repair and transcriptional regulation. Recent developments indicate that BRCA1 is a multifunctional protein, and disruption of its transcriptional activity could be crucial for tumor development.

MUTATIONS IN *BRCA1* account for approximately 45% of families with high incidence of breast cancer and for the majority of families with high incidence of both breast and ovarian cancer[1]. Several lines of evidence indicate that BRCA1 is a tumor suppressor, but a role as a negative regulator of cell proliferation is yet to be unambiguously demonstrated. *BRCA1*-linked tumors arising in carriers of germ-line mutations display loss of heterozygosity in the *BRCA1* locus with retention of the mutant allele[2]. BRCA1 induces the expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/CIP1}$, causing cell-cycle arrest[3]. Conversely, inhibition of *BRCA1* expression with antisense oligonucleotides results in accelerated proliferation in a mammary epithelial cell line[4]. It is still not clear whether these effects of BRCA1 on cell proliferation correspond to a physiological function or represent a response to abnormal levels of the protein induced by experimental conditions.

Human *BRCA1* codes for an 1863-amino-acid nuclear protein (Fig. 1a) with no detectable similarity to known proteins, with the exception of a RING-finger domain located in the N terminus and two BRCT (BRCA1 C-terminal) domains in tandem (aa 1653–1736 and aa 1760–1855)[5,6]. The BRCT is a globular domain found in proteins involved in repair and cell-cycle control[6]. Most of the documented cancer-associated mutations (Breast Cancer Information Core; http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/) cause truncations of the C-terminal region, a highly evolutionarily conserved region of the protein comprising the BRCT domains, underscoring the importance of this region for function.

Brca1 disruption in mouse leads to early embryonic death[7,8], and cells isolated from $Brca1^{-/-}$ embryos were shown to accumulate chromosomal abnormalities[9]. Cell-biological and genetic experiments have implicated BRCA1 in the maintenance of genome stability and DNA repair. BRCA1 has been found in large complexes that contain proteins involved in DNA repair[10–12], and human cells lacking BRCA1 display high sensitivity to γ-irradiation[13]. In addition, BRCA1 seems to be required for efficient homologous recombination[14]. To date, the evidence implicating BRCA1 in a variety of DNA-repair processes are based largely on genetic experiments and do not reveal by which mechanism BRCA1 acts. Although many scenarios remain possible at this stage, it is plausible that these effects are indirectly mediated through transcription activation.

Presence of a transcriptional activation domain in BRCA1

An early hint that BRCA1 might be involved in transcriptional activation came from the observation that the C-terminal region has a high content of negatively charged residues[5]. Highly acidic regions usually correlate with the transactivation domain in several eukaryotic transcription factors[15]. The hypothesis that BRCA1 could act as a transcription factor was tested by making fusions to a heterologous DNA-binding domain (DBD) and measuring the activation of a

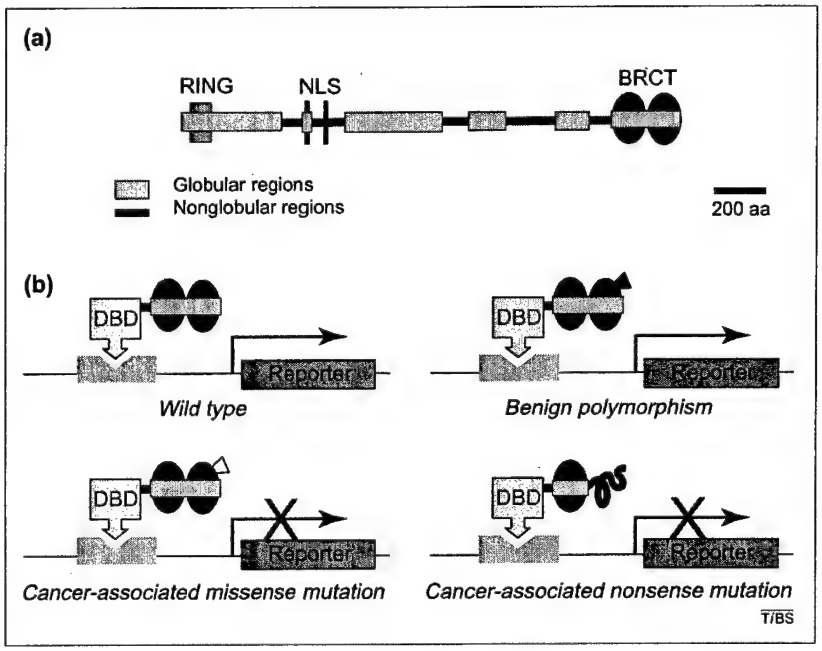

Figure 1

Transcriptional activation by BRCA1. **(a)** Domain structure of human BRCA1. Light-blue boxes and dark-blue lines correspond to predicted globular and nonglobular regions, respectively. The gray box represents the zinc-binding RING domain, and red circles represent the BRCA C-terminal (BRCT) domains. NLS, nuclear localization signal. Bar represents 200 amino acids. **(b)** Transcriptional activity by wild-type and mutant BRCA1 C-terminal region fused to a heterologous DNA-binding domain (DBD) (yellow boxes). Introduction of cancer-associated mutations (but not benign polymorphisms) abolishes reporter activation. Fusion to GAL4 DBD, a well-characterized heterologous DBD, enables the fusion protein to recognize specific sequences in the promoter of a reporter gene.

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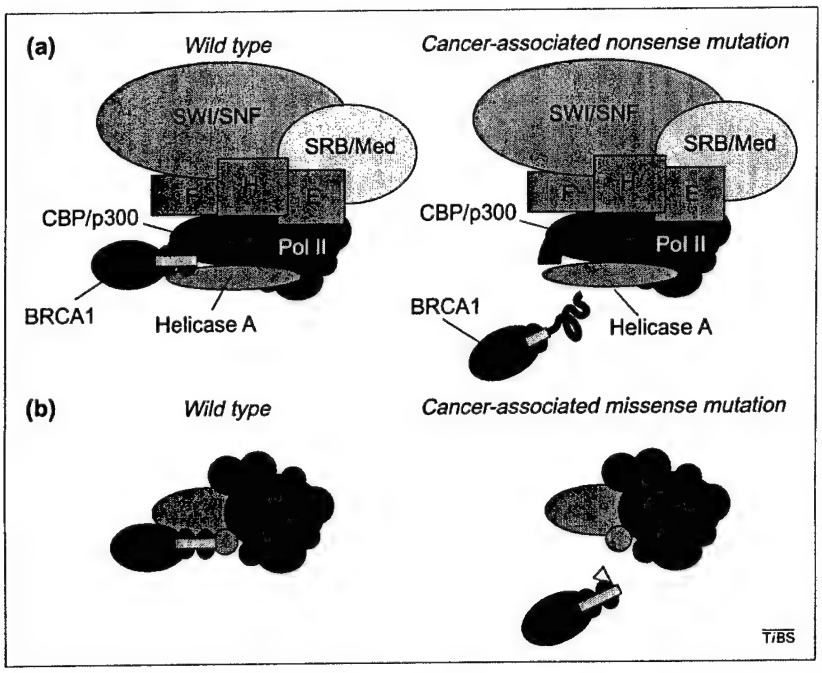

Figure 2

Interaction of BRCA1 with RNA polymerase II holoenzyme and with the core polymerase. **(a)** Physical interaction of BRCA1 with the RNA polymerase II holoenzyme via RNA helicase A and CBP/p300. The holoenzyme also contains the core polymerase subunits (dark-green circles), general transcription factors (blue squares), the SRB–Med complex and the chromatin remodeling SWI/SNF complex. Introduction of cancer-associated mutations abolishes interaction with RNA helicase A. **(b)** Physical interaction of BRCA1 with components of the core RNA polymerase II subunits, hRPB2 and hRPB10α (light-green circles). Introduction of cancer-associated mutations (open triangle) abolishes interaction with the core RNA polymerase II.

reporter gene by the fusion protein[16,17] (Fig. 1b). This kind of analysis is possible due to the modular nature of transcription factors in which DBDs and transactivation domains (domains that recruit the general transcription factors and RNA polymerase II) can function independently[15]. Results showing that the BRCA1 C terminus (aa 1560–1863) has the ability to activate transcription when fused to a GAL4 DBD (Refs 16,17) provided the initial experimental evidence that BRCA1 could be involved in transcription and pointed to a role for the BRCT domains. Fusion to GAL4 DBD, a well-characterized heterologous DBD, enables the fusion protein to recognize specific sequences in the promoter of a reporter gene and is used when a DNA-binding sequence for a particular protein is unknown (Fig. 1b).

Assays involving overexpression of fusions of heterologous DBD with truncated proteins can generate artifactual results. In addition, transcription activation could be a physiological function of BRCA1 but still be unrelated to its role in tumor suppression. Introduction of cancer-associated mutations abolished transactivation[16,17], whereas benign polymorphisms did not[18] (Fig. 1b), suggesting that transactivation function is important to prevent tumor development. Extensive mutagenesis analysis of the C-terminal region confirmed that the BRCT domains correspond to the core transactivation domain of BRCA1 (Ref. 19). The fact that cancer-associated mutations result in loss of transcriptional activation function, in particular those that are not predicted to disrupt the BRCT fold, provided a genetic framework to link the transcriptional activation function of BRCA1 to the development of cancer. However, although BRCA1 can act as a transcriptional regulator, it is unclear which precise biochemical function is performed by BRCA1.

BRCA1 interacts with RNA polymerase II

Mammalian RNA polymerase II (pol II) has been shown to exist in two distinct multiprotein complexes: the 'core', composed of 12 RNA pol II subunits (~500 kDa), and the 'holoenzyme' (>1 MDa), containing the core plus the SRB–Med complex [this complex was isolated both genetically, as suppressors of RNA pol II deletions in yeast (SRB), and biochemically, as proteins required for activated transcription in a partially purified system *in vitro*], general transcription factors and factors involved in chromatin remodeling (Fig. 2). Support for a physiological role of BRCA1 in transcription came from results showing that BRCA1 co-purified with pol II holoenzyme under conditions in which other transcription factors such as YY1, TFII-I, p65 Rel and RBPJ-κ did not[20] (Fig. 2a). BRCA1 interacts directly with RNA helicase A, a component of the holoenzyme that also interacts with the coactivator CBP/p300 (Ref. 21). Importantly, an ectopically expressed epitope-tagged BRCA1 carrying a cancer-associated mutation failed to interact with the holoenzyme[20]. Interestingly, GAL4 DBD–BRCA1 C terminus fusions can activate transcription *in vitro* in the presence of core pol II (Ref. 22; Fig. 2b). This *in vitro* system was later used, in transcription reconstitution experiments, to define hRPB10α (a homolog of yeast ABC10 α shown to be essential in yeast) and hRPB2 (homologous to the prokaryotic pol II β subunit) as the interaction partners in the core polymerase[23]. Recently, it was shown that the coactivator CBP/p300 interacts with and acts as a coactivator for BRCA1 (Ref. 24) (see Table 1 for BRCA1-interacting proteins involved in transcription). The interaction does not seem to be mediated by RNA helicase A, because a GST-tagged CBP fragment (aa 451–721) lacking a RNA-helicase-A-binding site (aa 1805–1890) can co-precipitate BRCA1 (Ref. 24). Although the results discussed so far revealed a function for BRCA1 in transcription, and the *in vitro* experiments suggest potential mechanisms through which BRCA1 activates transcription, they provide no clear idea of the physiological outcome of BRCA1-mediated transcription.

Transcriptional activation by BRCA1

In 1997, experiments showing that ectopically expressed wild-type BRCA1 caused cell-cycle arrest via transactivation of the cell-cycle inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner suggested a basis for BRCA1 tumor suppressor action and provided the first hint of putative downstream effectors of BRCA1 (Ref. 3; Fig. 3a). Importantly, cancer-predisposing, transactivation-deficient mutants failed to cause either cell-cycle arrest or $p21^{WAF1/CIP1}$ induction. However, the experiments did not discriminate between a direct or indirect effect of BRCA1 on transactivation of $p21^{WAF1/CIP1}$.

It was also found that BRCA1 physically interacts with p53 and enhances p53-mediated activation of p53-responsive genes including $p21^{WAF1/CIP1}$, suggesting a more direct effect of BRCA1 on transcription[25,26] (Fig. 3a). These results are important, because they link the p53 pathway and BRCA1 function and raise intriguing questions. Does binding of BRCA1 to p53 induce changes in p53 DNA-binding affinity for different p53-responsive elements, therefore conceivably shifting p53 response from a set of target genes to another? To demonstrate a direct involvement of BRCA1 in the activation, it would also be important to test if antibodies against BRCA1 can induce supershift of p53-containing complexes in an electromobility shift assay (EMSA). In EMSA, nuclear extracts are incubated with a radiolabeled DNA probe containing the sequence-specific binding site. Proteins that recognize and bind the probe appear as a labeled band on a gel. The identity of the protein is determined by adding a specific antibody that, upon binding to the protein–DNA complex, will retard its migration (mobility shift) on a gel.

In a different system, BRCA1 was implicated in enhancing IFN-γ-stimulated growth arrest, most likely by binding to activated signal transducer and activator of transcription 1 (STAT1)[27] (Fig. 3b). STAT1 is phosphorylated by the Janus kinase (JAK) and mediates the biological effects of IFN-γ. In cells stimulated with IFN-γ, growth arrest is mediated by induction of $p21^{WAF1/CIP1}$ via an IFN-γ-responsive element. Experiments in HCC1937, a cell line carrying only one copy of a truncated version of BRCA1, indicate that BRCA1 is required for IFN-γ-mediated $p21^{WAF1/CIP1}$ induction[27]. BRCA1 differentially regulates some IFN-γ target genes by enhancing its growth-arrest response, but not other IFN-γ target genes such as IRF-1, SMAD7 and IP10, suggesting that BRCA1 displays promoter selectivity (Fig. 3b).

Lately, investigators have undertaken a global approach to define BRCA1 target genes using cDNA arrays. Inducible expression of BRCA1 in cultured cells led to programmed cell death and revealed that a major BRCA1 target gene is the DNA-damage-responsive gene *GADD45* (Ref. 28). Induction of *GADD45* has been shown to trigger JNK/SAPK-dependent apoptosis[29]. JNK/SAPK (Jun N-terminal kinase/stress-activated protein kinase) is a member of the mitogen-activated protein kinase (MAPK) family used by cells to relay signals triggered by growth factors and extracellular stimuli. Concomitant inhibition of JNK/SAPK by ectopic expression of a dominant-negative mutant of its upstream regulator SEK1 prevented BRCA1-induced apoptosis but not *GADD45* induction, indicating that BRCA1 induces apoptosis via JNK/SAPK. A similar approach using adenovirus-mediated BRCA1 infection also showed induction of cell-cycle-controlling genes (e.g. $p21^{WAF1/CIP1}$) and DNA-damage-response genes (e.g. *GADD45*) but failed to demonstrate induction of apoptosis, suggesting that this effect could be cell-type specific[30]. One caveat of these experiments is the fact that most of them, but not all, rely on overexpression of the protein in cells that express endogenous BRCA1. Establishment of HCC1937 cells carrying a stably transfected inducible BRCA1 will provide a powerful tool for these analyses. Also, as in other experiments involving *in vivo* expression of BRCA1, we cannot distinguish whether the effect on transcription is direct or indirect.

Table 1. BRCA1-interacting proteins involved in transcription

Interacting protein	BRCA1-binding site	Function of interacting protein	Refs
BARD1	RING finger[a]	Unknown; repair (?), mRNA processing (?)	37,44
CBP/p300	1–303 and 1314–1863[b]	Transcriptional coactivator	24
CtiP	1651–1863[a,c]	Binds to CtBP transcription repressor	33,34
HDAC1 and HDAC2	1536–1863[d]	Histone deacetylases involved in chromatin remodeling	32
c-Myc	433–511[e]	Helix-loop-helix transcription factor	31
p53	224–500[e]	Transcription activation	25,26
Rb	1536–1863[f]	Retinoblastoma tumor suppressor gene. Interacts with E2F and represses E2F-mediated transcription	32
RbAp46 and RbAp48	1536–1863[g]	Rb-interacting protein. Component of histone deacetylase complexes involved in chromatin remodeling	32
RNA helicase A	1650–1800[h]	Component of the RNA polymerase II holoenzyme. Also interacts with CBP/p300	21
RPB2	1560–1863[i]	Component of the core RNA polymerase II. Homolog of bacterial RNA polymerase β subunit	23
RPB10α	1560–1863[i]	Component of the core RNA polymerase II. Homolog of yeast ABC10α	23
STAT1α	502–802[j]	Transcription activation induced by interferon	27

Binding site was identified by: [a]Mammalian two-hybrid assay; [b]Binding of glutathione-*S*-transferase (GST)–CBP to Myc-tagged BRCA1 fragments in pull-down assays; [c]Yeast two-hybrid assays; [d]Binding to GST–BRCT in pull-down assays; [e]Interaction of *in vitro* translated BRCA1 fragments with GST–p53 and GST–BRCA1 fragments in pull-down assays; [f]Interaction of *in vitro* translated BRCA1 fragments with GST–RB; [g]Interaction of *in vitro* translated BRCA1 fragments with GST–RbAp; [h]Interaction of *in vitro* translated RHA with GST–BRCA1 fragments; [i]Interaction of biotin-binding PinPoint domain fusion of BRCA1 to core polymerase; [j]*In vitro* binding assay using GST–BRCA1 fragments.
Abbreviations: BARD1, BRCA1-associated RING-domain protein 1; BRCT, BRCA1 C-terminal domain; CBP, CREB-binding protein; CtiP, CtBP-interacting protein; HDAC, histone deacetylase; Rb, retinoblastoma; RbAp, retinoblastoma-associated protein; STAT, signal transducer and activator of transcription.

Transcriptional repression by BRCA1

The data discussed above show that BRCA1 can activate transcription, but it might also be able to repress transcription. In a yeast two-hybrid approach, BRCA1 was shown to interact with the helix-loop-helix transcription factor c-Myc (Ref. 31) (Fig. 3c). In addition, co-transfection studies demonstrated that BRCA1 repressed Myc-mediated transcription and suppressed the number of transformed foci in Ras- or c-Myc-transformed embryo fibroblasts. These results suggest that BRCA1 can negatively regulate transcription by c-Myc. Because overexpressing a particular protein might repress the activity of a transcription factor through physiologically irrelevant protein–protein interactions, it is important to establish whether BRCA1 deficiency in cells facilitate c-Myc-mediated transformation.

The fact that BRCA1 has a transcriptional activation domain might seem paradoxical with its function in repression, but other lines of evidence suggest that BRCA1 might be able to modulate transcription in a context-dependent way. When a cDNA expression library was probed with a histidine-tagged BRCT domain in a far-western screen, one retinoblastoma (Rb)-binding protein, RbAp46 (a component of histone deacetylase complexes), was obtained[32] (far-western analysis uses a protein probe to detect interactions with

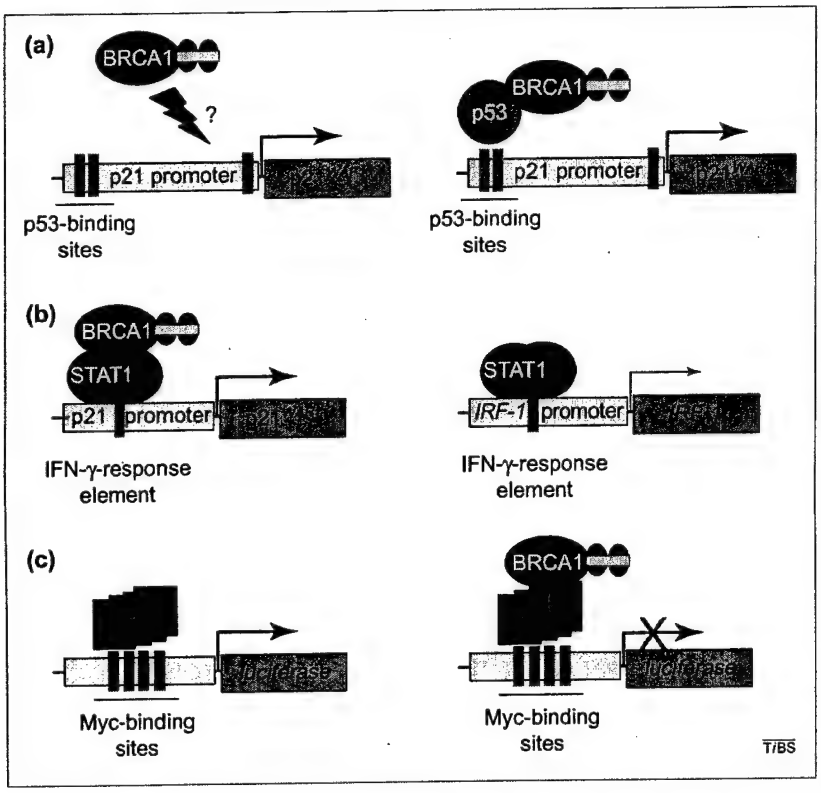

Figure 3

Transcriptional regulation by BRCA1. Functional interaction of BRCA1 with other DNA-binding proteins. **(a)** BRCA1 is able to activate the $p21^{WAF1/CIP1}$ promoter (yellow box) in a p53-dependent (right panel) and -independent (left panel) manner targeting two different regions of the promoter. **(b)** Selectivity of BRCA1 coactivation of STAT1 (signal transducer and activator of transcription 1; green circles). BRCA1 coactivates STAT1-mediated transcription in the $p21^{WAF1/CIP1}$ promoter (left panel, yellow box) but not the *IRF-1* promoter (right panel, yellow box). **(c)** BRCA1 repression of Myc-mediated transcription of a reporter gene.

proteins immobilized on filter membranes). Other proteins in the Rb tumor suppressor pathway, such as RbAp48 and Rb itself, are also capable of interacting with BRCA1. Similarly, the C-terminal region of BRCA1 was shown to bind to the histone deacetylases HDAC1 and HDAC2 *in vitro*[32].

Two independent approaches to identify BRCA1-binding proteins, one using the yeast two-hybrid system and the other using the Sos-recruitment system (a variation of the two-hybrid system commonly used when the proteins to be tested display intrinsic transcriptional activity), resulted in the isolation of CtIP (CtBP-interacting protein), a co-repressor for different cellular transcription factors[33,34]. Cancer-associated mutations in BRCA1 abolished binding to CtIP (Refs 33,34), as did DNA damage[35].

Of particular importance to breast and ovary carcinogenesis are preliminary reports that BRCA1 modulates estrogen receptor α transcription[36], but further studies are needed to determine the physiological relevance of this modulation. It is possible that BRCA1 also regulates promoter selectivity of ER α target genes.

A recent report shows that BRCA1 and its RING-finger-interacting protein BARD1 are present in a complex containing the cleavage-stimulating factor CstF50, which helps specify the site of mRNA processing during polyadenylation[37]. Whereas addition of a monoclonal antibody against BARD1 specifically enhanced the cleavage reaction *in vitro* and suggests that BARD1 antagonizes 3′-end formation, the role of BRCA1 is unclear[37].

It is still too early to judge the significance of the interactions of BRCA1 with different proteins (Table 1), but they provide indirect evidence of its role in transcription and suggest that BRCA1 can switch from a positive to a negative regulator in different contexts. The next step will be to investigate the physiological relevance of these interactions by complementary methods.

Transcription-coupled repair: a unifying theme?

Although it is possible that BRCA1 has two separate functions in DNA repair and in transcription regulation, it is also conceivable that the role of BRCA1 in DNA repair is mediated by transcriptional regulation. In agreement with the latter notion, intact BRCA1 seems to be required for transcription-coupled repair (TCR) of oxidative DNA damage but not for its global removal in embryonic stem cells from $Brca1^{-/-}$ mice[38]. Experiments with human BRCA1-deficient cells also suggest that BRCA1 is required for TCR (Ref. 39). TCR, also called preferential repair of the transcribed strand, refers to the fact that genes being actively transcribed are repaired more rapidly than other genes in the genome, and rapid repair is confined to the transcribed strand only. It is believed that one of the components of the RNA pol II-associated general factors, TFII-H, can shift from a transcription to a repair mode in the stalled RNA pol II (Ref. 40). Could BRCA1 also display such a switch? We should keep in mind that the data do not reveal whether BRCA1 is directly required for TCR or rather necessary for the transcription of genes required for TCR.

One way to test the hypothesis that the observed role of BRCA1 in repair is an indirect effect of its transcriptional function is to examine the cancer-predisposing alleles of BRCA1 and their outcome in functional assays for transcriptional activation and for DNA repair activity. With the recent progress on functional assays with BRCA1 for both of these functions, this approach might now be possible[19,41]. If all mutations that affect transcriptional activation also affect its DNA-repair function, this would provide strong evidence that it is the same biochemical pathway. To be informative, these mutations should not lead to instability of the mRNA, or the protein, or lead to drastic disruption of the folding. Although many mutations have been recorded in the C-terminal region of BRCA1, only a few have been characterized as either benign polymorphisms or cancer-associated mutations. It is expected that the lack of genetic data regarding cancer predisposition of BRCA1 alleles will be balanced by knowledge of the crystal structure of the BRCT domain of XRCC1 (a scaffolding protein in the mammalian base excision repair pathway), which allowed modeling of the BRCA1 BRCT domain and prediction of structural changes in the protein

caused by different mutations[42]. Unfortunately, until further structural data emerge for other regions of the protein, the analysis will be restricted to the BRCT and RING-finger domains. Another possible approach to the same problem could be the use of reciprocal mutants on components of the RNA pol II that restore binding and function to a mutant transcription factor. An analogous approach has been used successfully to probe TBP–TFIIB interaction[43]. In this case, it would be possible to design reciprocal mutants in pol II components that restore binding of mutant BRCA1. The prediction would be that if transcription is a necessary and sufficient step in processing repair, then, in this context, a mutant BRCA1 will be able to perform DNA repair. If, however, the reciprocal mutation is then able to sustain transcription but not repair, we will have strong evidence that the two functions assigned for BRCA1 are separate. Certainly, any outcome will reveal exciting new avenues of research.

Future perspectives

Is BRCA1 a transcription factor or a coactivator? As we have seen, BRCA1 contacts the RNA polymerase II holoenzyme components p300/CBP, RNA helicase A, RPB10α and RPB2. The fact that RPB10α is a common component of all three RNA polymerases raises the possibility that BRCA1 might also regulate pol I and pol III transcription, and it will be important to see if BRCA1 is also present in these complexes. However, to date, no one has been able to demonstrate sequence-specific binding to DNA by BRCA1, although several instances where BRCA1 functionally interacts with other DNA-binding proteins have been reported.

What is the role of BRCA1 in the RNA pol II holoenzyme? Crude stoichiometric estimates indicate that BRCA1 is only present in a subpopulation of the holoenzyme[20], suggesting that, instead of a general role in transcription, BRCA1 might be present in only a fraction of pol II possibly involved in transcription of a particular subset of genes. Alternatively, BRCA1 might only interact with holoenzyme complexes engaged in transcription. The *in vitro* transcription reconstitution assays can be used to distinguish between these possibilities. It might be feasible to immunodeplete BRCA1-containing holoenzyme complexes and compare the activities of different holoenzyme preparations.

Are BRCA1-target genes the same in different tissues? Because tissue specificity for tumor formation cannot be explained by expression patterns (as BRCA1 is ubiquitously expressed), it is plausible that BRCA1 might be required for a subset of genes that are highly transcribed in certain tissues such as breast and ovary. Therefore, lack of functional BRCA1 would prime cells in these tissues to transformation. In light of the data on the involvement of BRCA1 in TCR, this idea is particularly appealing. It will be crucial to determine which biochemical step in TCR requires BRCA1.

The evidence for the role of BRCA1 in transcriptional regulation can be summarized as follows: (i) the C terminus of BRCA1 acts as a transcriptional activation domain when fused to a heterologous DNA-binding domain; (ii) BRCA1 can be found in complex with the RNA polymerase II (core and holoenzyme); (iii) ectopic expression of BRCA1 induces transcription from a variety of different promoters; and (iv) several BRCA1-interacting proteins have well-characterized roles in transcription. The strong correlation of cancer-associated mutations and its loss of function phenotype in the experiments described strengthen the idea that the role of BRCA1 in transcription is physiologically relevant during the development of the disease. Many questions remain unanswered, but the biochemical and genetic approaches discussed here form the basis to attribute a definite biological function for BRCA1.

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Gene context conservation of a higher order than operons

Warren C. Lathe III, Berend Snel and Peer Bork

Operons, co-transcribed and co-regulated contiguous sets of genes, are poorly conserved over short periods of evolutionary time. The gene order, gene content and regulatory mechanisms of operons can be very different, even in closely related species. Here, we present several lines of evidence which suggest that, although an operon and its individual genes and regulatory structures are rearranged when comparing the genomes of different species, this rearrangement is a conservative process. Genomic rearrangements invariably maintain individual genes in very specific functional and regulatory contexts. We call this conserved context an uber-operon.

THE REGULATORY AND neighborhood context (Box 1) of most genes is seen as generally fluid[1,2]. A study of 11 genomes[3] showed that the gene order and content of nearly all the known operons of the *Escherichia coli* and *Bacillus subtilis* genomes are either missing or incomplete in other species, suggesting that genomes are randomly rearranged.

A few genes are known to have highly conserved neighborhoods in many bacterial genomes[4,5]. For example, the *rplC* and *rplD* ribosomal genes are invariably found immediately adjacent to each other in the 22 bacterial genomes completed as of submission of this article. It has been suggested that such highly conserved neighbors are due to the fact that the products of these genes interact physically or functionally[6,7]. And yet, these conserved pairs of genes are the exception rather than the rule and, even when the gene order within an operon is conserved, regulation can be distinct in different species[8]. The regulatory and neighborhood context of most genes varies greatly when comparing genomes of different species.

This apparent fluidity masks a greater conservation of regulatory and neighborhood context. In reality, the different operon architectures seen from genome to genome result from very conservative rearrangements of genes. Examples from three disparate cellular systems (translational machinery, flagellar structure and chemotaxis, and ABC transporter genes) show that, although genomic rearrangements cause variation in the immediate neighborhood of a gene, many genes are maintained over evolutionary time within the context of a discrete set of functionally related genes. We call this set of genes that is conserved at a higher level of organization an uber-operon.

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A translation-associated uber-operon

The exploitation of context information, such as neighborhood, is becoming an important tool in function prediction based on genomic sequence[5–7,9–12]. However, as mentioned above, only a few neighborhood relations and operons are strictly conserved over a wide range of species[4,5]. This is also true for ribosomal operons[13,14], despite the sequence conservation of the individual genes therein. Wachtershauser suggested that the ribosomal gene cluster in the 'universal ancestor' was broken up into smaller clusters during evolution[13]. We propose that the picture is somewhat more complex. Rather than the break up of a large ancestral gene cluster, the evolution of the ribosomal clusters appears to have involved the joining of clusters, break up into smaller clusters and the rearrangement of these into new clusters in a conservative fashion.

The extent of conservative operon reassortment can be illustrated by the neighborhood of one translation-associated gene (*tufA*) that is always found within ribosomal operons. This gene codes for an elongation factor involved in translation and was originally described as part of the *str* operon in *E. coli*[15]. Although the neighborhoods vary for *tufA* in the different genomes, *tufA* invariably occurs together with ribosomal and other translation-associated genes (Fig. 1). It is not only found in the putative ancestral neighborhood[13] of *rpsJ* and *fusA* but also in the neighborhood of different translation-associated genes including *rpmG* (*Helicobacter pylori*), *secE* (*Chlamydia pneumoniae*) and others (*Mycoplasma genitalium*, *Methanococcus jannaschii*). There were no exceptions to a translation-associated neighborhood for *tufA* in any of the 15 genomes studied here.

Phylogenetic analysis of the genes displayed in Fig. 1 does not indicate any detectable lateral transfer of genes across divergent species boundaries[13] (W.C. Lathe, unpublished). From this

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Invited Review

A nuclear function for the tumor suppressor BRCA1

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Summary. The breast and ovarian cancer susceptibility gene *BRCA1* has been recently cloned and revealed an open reading frame of 1863 amino acids, but a lack of significant homology to any known protein in the database has led to few clues about its functions. One of the first steps to investigate the function of BRCA1 was to define its subcellular localization. Several reports have led to contradictory findings that include: nuclear localization in normal cells and cytoplasmic in breast and ovarian cancer cells; nuclear in both normal and cancer cells; cytoplasmic and secreted to the extracellular space; present in tube-like invaginations of the nucleus; and colocalizing with the centrosome. As is apparent, the subcellular localization has been the most controversial aspect of BRCA1 biology and is a key point to uncover its functions. In this paper we review the published data on subcellular localization of BRCA1 with special emphasis on the antibodies and techniques used. We conclude that there is now overwhelming evidence to support a nuclear localization for BRCA1, both in normal and cancer cells. In addition, several BRCA1-interacting proteins have been isolated and they are preferentially located in the nucleus. Evidence supporting a physiological function for BRCA1 during DNA repair and transcriptional activation is also discussed.

Key words: Breast cancer, Tumor suppressor gene, BRCA1, Antibody specificity

Abbreviations: GST: Glutathione-S-transferase; NLS: nuclear localization signal; CEFs: chicken embryo fibroblasts; SDS: sodium dodecyl sulfate; DAPI: 4',6-diamidino-2-phenylindole; FBS: fetal bovine serum; PBS: phosphate buffered saline

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Introduction

Mutations in *BRCA1* account for approximately 45% of the families with high incidence of breast cancer and 80% of families with high incidence of both breast and ovarian cancer (Easton et al., 1993). Identification of human *BRCA1* by positional cloning techniques revealed an open reading frame coding for 1863 amino acids with no statistically significant homology to proteins in the database, with the exception of a zinc-binding RING finger motif (C3HC4) in the N-terminal region (Miki et al., 1994). This motif is found in several proteins that have their functions mediated through DNA binding (Saurin et al., 1996). In addition, the presence of two putative nuclear localization signals (NLSs; aa 500-508 and 609-615) and an excess of negatively charged residues in the C-terminal region of BRCA1 suggested a function for *BRCA1* in transcriptional regulation (Miki et al., 1994). Using a different computational strategy, Koonin et al. partitioned the BRCA1 sequence into putative globular and non-globular domains and used the globular domains to perform iterative searches in the database (Koonin et al., 1996). These studies defined a globular domain repeated in tandem in the C-terminal region of BRCA1, named BRCT (for BRCA1 C-terminal domain) also present in 53BP1, a p53 binding protein (Koonin et al., 1996). This study was later extended and defined a superfamily of proteins containing the BRCT domains involved in DNA damage and cell cycle checkpoints (Bork et al., 1997; Callebaut and Mornon, 1997). Collectively, the fact that the RING finger, the NLSs, the excess of negatively charged residues and the BRCT domains are conserved in human, dog, rat and mouse *Brca1*, suggests that these regions are significant for BRCA1 function (Abel et al., 1995; Lane et al., 1995; Sharan et al., 1995; Szabo et al., 1996; Bennet et al., 1999).

Is BRCA1 aberrantly localized in breast and ovarian cancer?

One of the first strategies to understand the biochemical function of BRCA1 was immunofluorescence

and immunocytochemical analysis. Chen et al. (1995a,b) initially characterized BRCA1 as a 220 kDa nuclear phosphoprotein in normal cells as well as in cells derived from tumors other than breast and ovarian cancer. Interestingly, in the majority of breast and ovarian cancer cell lines and cells obtained from malignant pleural effusions of these tumors, BRCA1 appeared to be mislocalized to the cytoplasm (Chen et al., 1995a,b). Previous experiments raised questions about the role of BRCA1 in sporadic breast and ovarian cancer since no mutations in BRCA1 had been found in sporadic breast cancers and very few in ovarian cancers (Futreal et al., 1994). Mislocalization of BRCA1 to the cytoplasm suggested that a deficient nuclear transport mechanism might disrupt BRCA1 function in sporadic tumors in the absence of loss-of-function mutations. However, these authors only performed biochemical fractionation in HBL100 cells, and there was no fractionation data of the cancer cell lines showing mislocalization (Chen et al., 1995a,b).

In 1996, Scully et al. (1996a) reexamined the question by using an affinity purified polyclonal antibody as well as seven monoclonals (for a comprehensive list of BRCA1 antibodies published in the literature see Table 1; for a comparative study of several antibodies see Wilson et al., 1999) raised against various epitopes and found a consistent "nuclear dot" pattern in cell lines fixed with neutral paraformaldehyde, or methanol, or 70% ethanol. Moreover, biochemical fractionation analysis of three cancer cell lines (SKOV-3, MCF-7 and U20S) confirmed the presence of BRCA1 in the nuclear but not in the cytoplasmic fractions (Scully et al., 1996a). Although some of the antibodies showed weak cytoplasmic staining, confocal microscopy studies could not demonstrate colocalization of the signals derived from different antibodies, strongly arguing for non-specific cross-reactivity in the cytoplasm. Paraffin-embedded sections fixed with alcoholic formalin were shown to generate nuclear, both nuclear and cytoplasmic, as well as cytoplasmic staining. However, when these sections were treated with microwave heating, the staining was predominantly cytoplasmic suggesting that artifacts due to sample preparation may contribute to the confusion in BRCA1 location. In fact, cell lines where BRCA1 had been shown to be nuclear both by subcellular fractionation of unfixed cells and by immunostaining, displayed variable results when subjected to different fixation and heating conditions (Scully et al., 1996a).

To circumvent specificity problems with antisera to native BRCA1 epitopes, Chen et al. (1995b) ectopically overexpressed an N-terminal FLAG-tagged BRCA1 to show the tagged protein to be in the nucleus of normal cells but in the cytoplasm in a series of breast cancer cell lines. A caveat of ectopic overexpression is that high levels of protein can saturate subcellular compartments and result in the presence of the protein where it is not normally found under physiological conditions. Moreover, overexpression of BRCA1 can also cause toxicity and induce changes in cell morphology (Wilson et al., 1997). An important issue that remains is the localization of BRCA1 in rapidly proliferating cells versus contact-inhibited cells. Contact-inhibited cells have very low, in many cases undetectable, BRCA1 levels (Chen et al., 1996a; Jin et al., 1997). This may also be a source of artifactual results because antibodies that show both nuclear and some cytoplasmic staining will show only cytoplasmic staining when cells are contact inhibited due to the absence BRCA1 and presence of cross-reacting species in the cytoplasm. Examining the published results, cells presenting cytoplasmic staining are found in close contact with other neighboring cells. Immunostaining of cells expressing BRCA1 (HBL100) and chicken embryo fibroblasts (CEFs) that lack BRCA1 judged by low stringency southern blots (Miki et al., 1994), is a particularly revealing example of cytoplasmic cross-reactivity (Fig. 1A). Cells expressing BRCA1 (HBL100), show both a nuclear dot pattern and a diffuse cytoplasmic staining, whereas cells lacking BRCA1 (CEFs) show only a diffuse cytoplasmic staining. Moreover, to confirm the absence of BRCA1 in CEFs, immunoprecipitations were performed with cellular extracts obtained sequentially with (i) a mild Triton-containing buffer (HNTG) that extracts cytosolic proteins and (ii) subsequently with a harsher SDS-containing buffer (RIPA) (Fig. 1B). Typically, these studies confirm that BRCA1 is present only in the nuclear fraction and not in the cytoplasm of HBL100 and MCF-7 cells. In addition, immunoreactivity against BRCA1 is not observed CEFs, confirming the absence of BRCA1 in these cells.

Is BRCA1 a granin?

The discovery of a granin sequence in BRCA1 suggested that BRCA1 and BRCA2 might be secretory proteins (Jensen et al., 1996a). Granins are a family of highly variable proteins that share a 10 amino acid motif and participate in secretory pathways (Ozawa and Takata, 1995). Interestingly, using polyclonal antibodies detected a 190 kDa in cell lysates and a 180 kDa protein from baculovirus lysates expressing the recombinant protein rather than the typical 220 kDa protein (Table 1). Furthermore, the majority of the reacting species localized to the membrane fraction and a small amount to the cytoplasm. The staining of primary human mammary epithelial cells revealed a granular pattern in both nucleus and Golgi complex (Jensen et al., 1996a). Later, using confocal microscopy Coene et al. suggested that the nuclear dot pattern represents cross-sections of cytoplasmic invaginations and that BRCA1 was mostly perinuclear (Coene et al., 1997). Although antibodies cross-reactivity may explain Golgi staining, additional evidence for BRCA1 being secreted is still lacking.

Several observations, following this initial publication, rebut the idea that BRCA1 is a granin. That includes the fact that the antibodies (Table 1) used in the

Table 1. α-BRCA1 antibodies.

ANTIBODY	BRCA1 EPITOPE[a]	USES	LOCALIZATION (REFERENCE)[b]	PROTEIN SIZE[c] AND OTHER COMMENTS
α-BRCA1	(G) 762-1315	IP[d], IB, IF	Nuclear in normal cells and cells from tumors other than breast and ovary and cytoplasmic in breast and ovarian cancer cells (Chen et al., 1995a,b).	220 kDa (Chen et al., 1995a,b). Mouse polyclonal
α-BRCA1	860-881	IF, IB	Cytoplasmic tube-like invaginations of the cytoplasm in both normal and breast cancer cells (Coene et al., 1997).	190, 220 and 240 kDa (Coene et al., 1997). Rabbit polyclonal.
α-BRCA1	1848-1863	IF, IB	Cytoplasmic tube-like invaginations of the cytoplasm in both normal and breast cancer cells (Coene et al., 1997).	190, 220 and 240 kDa (Coene et al., 1997). Rabbit polyclonal.
α-BRCA1 BgI	(G) 341-748	IP	n.s.[e]	220 kDa (Chen et al., 1995). Mouse polyclonal.
α-BRCA1N	(G) 1-302	IP		220 kDa (Chen et al., 1996). Mouse polyclonal.
A	2-20	IB, IP	Nuclear and some cytoplasmic in fractionation experiments (Ruffner and Verma, 1997).	220 kDa (Ruffner and Verma, 1997; Wilson et al., 1999). Rabbit polyclonal.
A19	1847-1863	IP, IB	Nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996).	220 kDa (Scully et al., 1996). Rabbit polyclonal.
AP11	(G) 1313-1863	IP, IB, IF	n.s.	n.s. Mouse monoclonal.
AP12	(G) 1313-1863	IP, IB, IF	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
AP16	(G) 1313-1863	IP, IB, IF	nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996; Jensen et al., 1996b). Variable in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	220 kDa (Scully et al., 1996; Wilson et al. 1999). Mouse monoclonal.
B	70-89			220 kDa (Ruffner and Verma, 1997). Rabbit polyclonal.
B112	(G) 2-355	IF, IB, IP	Diffuse nuclear staining and absent from nucleoli (Wilson et al., 1997).	230 kDa (Wilson et al., 1997); 220 kDa (Wilson et al., 1999). Rabbit polyclonal.
BPA-1	8-475	IB	n.s.	220 kDa (Thomas et al., 1996). Rabbit polyclonal.
BPA-2	1293-1863	IB, IF	Nuclear dot pattern both in normal and cancer cell lines (Thomas et al., 1996).	220 kDa (Thomas et al., 1996). Rabbit polyclonal
BR64		IF	Nuclear dot pattern (Jensen et al., 1998).	220 kDa (Jensen et al., 1998). Rabbit monoclonal, available through Upstate Biotechnology.
C	768-793	IB, IP, IF	n.s.	220 kDa (Ruffner and Verma, 1997; Wilson et al., 1999). Rabbit polyclonal.
C-19	1844-1863	IP	n.s.	180-190 kDa (Jensen et al., 1996a)[f]. Rabbit polyclonal.
C-20	1843-1862	IP, IF, IB,	Predominantly granular cytoplasmic, with nuclear and Golgi staining (Jensen et al., 1996a). Nuclear and associated with the centrosome during mitosis (Hsu and White, 1998). Nuclear (Thakur et al., 1997; Wilson et al., 1999).	220 kDa (Chen et al., 1995; Thomas et al., 1996; Thakur et al., 1997; Wilson et al., 1999); 180-190 kDa (Jensen et al., 1996a,b)[f]; 230 kDa (Wilson et al., 1996); 185 kDa (Gudas et al., 1995). Rabbit polyclonal, available through Santa Cruz Biotechnology. Cross-reacts with EGFR and HER2 (Wilson et al., 1996).
D	1847-1863	IB, IP	Nuclear and some cytoplasmic in fractionation experiments (Ruffner and Verma, 1997).	220 kDa (Ruffner and Verma, 1997). Rabbit polyclonal
D-20	1-20	IB, IF	n.s.	190 kDa (Jensen et al., 1996a); 220 kDa (Wilson et al., 1999). Rabbit polyclonal, available through Santa Cruz Biotechnology. Does not cross-react with EGFR and HER2 (Wilson et al., 1996).

continued

GLK2	1839-1863	IF	Cytoplasmic in cell lines. Cytoplasm or no signal in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	Mouse monoclonal.
I-20	1823-1842	IB	n.d.[g]	230 kDa (Wilson et al., 1996). Rabbit polyclonal, available through Santa Cruz Biotechnology. Does not cross-react with EGFR and HER2 (Wilson et al., 1996).
Ki-8	903-919	IP, IB	Nuclear in fractionation experiments (Zhang et al., 1997).	215 kDa (Zhang et al., 1997). Mouse monoclonal.
M-20	mouse BRCA1 C-terminus	IB, IF	n.d.	215 kDa (Zhang et al., 1997). Goat polyclonal, available through Santa Cruz Biotechnology.
MS110	(G) 1-304	IP, IB, IF	Nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996, 1997b; Jensen et al., 1996b; Hsu and White, 1998; Wilson et al., 1999). Variable in paraffin-embedded cell pellets depending on conditions[h] used (Jensen et al., 1996b).	220 kDa (Scully et al., 1996a; Hsu and White, 1998; Scully et al., 1997b; Wilson et al., 1999). Mouse monoclonal, available as Ab-1 from Oncogene Research Products.
MS13	(G) 1-304	IF, IP, IB	Nuclear dot pattern both in normal and cancer cell lines. Variable in paraffin-embedded cell pellets depending on conditions used (Scully et al., 1996, 1997b; Jensen et al., 1996b)	220 kDa (Scully et al., 1996, 1997b; Wilson et al., 1999). Mouse monoclonal, available as Ab-2 from Oncogene Research Products.
N25	1-25	IF	Cytoplasmic in cell lines. Cytoplasm or no signal in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	Mouse monoclonal.
SD112	(G) 758-1313	IP, IB	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
SD118	(G) 758-1313	IP, IB, IF	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
SD123	(G) 758-1313	IP, IB, IF	n.s.	220 kDa (Chen et al., 1998; Wilson et al., 1999). Mouse monoclonal.
SG11	(G) 1847-1863	IF, IP	Nuclear dot pattern both in normal and cancer cell lines. Variable in paraffin-embedded cell pellets depending on conditions used (Scully et al., 1996; Jensen et al., 1996b).	Mouse monoclonal, available as Ab-3 from Oncogene Research Products.
ZB1	(G) 13-75	IB, IP	Nuclear with some amount cytoplasmic in fractionation experiments (Aprelikova et al., 1996).	220 kDa (Aprelikova et al., 1996). Rabbit polyclonal.
6B4	(G) 341-748	IP, IB		220 kDa (Chen et al., 1996a; Aprelikova et al., 1996). Mouse monoclonal.
17F8	(G) 762-1315	IP, IB, IF	Nuclear at lower (< 3 μg/ml) concentrations of antibody (Wilson et al., 1999).	220 kDa (Wilson et al., 1999). Mouse monoclonal, available through GeneTex.
24G11		IP, IB	n.d.	220 kDa (Chen et al., 1995b). Mouse monoclonal.
113	673-1365	IP, IB, IF	Nuclear (Wilson et al., 1999).	220 kDa (Wilson et al., 1999). Rabbit polyclonal.
115	673-1365	IP, IB, IF		220 kDa (Wilson et al., 1999). Rabbit polyclonal.
579	903-919	IP, IB	n.d.	215 kDa (Zhang et al., 1997). Rabbit polyclonal.

The references in this table are not extensive. Due to space limitations we have focused on the initial papers. A direct comparison of several monoclonal and polyclonal antibodies has been recently published (Wilson et al., 1999). [a]: all the antibodies have been raised against human BRCA1 epitopes unless otherwise stated. (G): antibody was raised against a GST-fusion protein. The remaining antibodies were raised using synthetic or recombinant peptides. [b]: In this table we have chosen to be conservative and not to consider experiments reported but not shown in the original papers. [c]: Although many antibodies described here showed several reactive bands, the size shown is that of the reactive species considered by the authors to represent the full length BRCA1. [d]: The uses described here were the ones reported in the original. We have not considered experiments reported but not shown. IP: immunoprecipitation; IF: immunofluorescence; IB, immunoblots. [e]: Not shown. [f]: 190 kDa species was detected from cell lysates and the 180 kDa species was detected from expressing the recombinant protein in baculovirus. [g]: Not done. [h]: Conditions vary in fixatives (neutral or alcoholic formalin) and in heat-induced epitope retrieval (no treatment; microwave or pressure cooker treatment).

majority of the experiments cross-reacts with human EGF Receptor and HER2, tyrosine kinase receptors that are frequently amplified in human breast and ovarian cancers (Wilson et al., 1996). Secondly, the statistical significance of the presence of the granin sequence in BRCA1 has been challenged, particularly allowing for a substitution in one of the motif's invariant position found in rat, dog and mouse Brca1 (Bradley and Sharan,

1996; Koonin et al., 1996). In addition, an alternative splice form of BRCA2 was found lacking the granin sequence, and shown by linkage analysis not to predispose to disease but to be a benign polymorphism (Mazoyer et al., 1996). Thirdly, the concept of BRCA1 being a tumor suppressor acting via the extracellular space has been challenged on the basis that it would be unlikely that a loss of heterozygosity in BRCA1 locus would cause a cell autonomous defect (Bradley and Sharan, 1996). In the scenario suggested by Jensen et al. (1996a), cells in breast and ovary tissue carrying no functional BRCA1 (after loss of hetero-zygosity) would still be bathed by the extracellular milieu containing BRCA1 secreted by the neighboring cells, making it unlikely to explain tumor initiation. To date, there has been no independent corroboration to the notion that BRCA1 is secreted.

The controversy resolved

The characterization of the putative nuclear localization signals (NLS) found in BRCA1 has been a key point to establish it as a nuclear protein (Miki et al., 1994). Two groups have identified slightly different NLSs [^{503}KRKRRP^{508}, ^{606}PKKNRLRRKS^{615} and ^{651}KKKKYN^{656} (Chen et al., 1996b); ^{501}KLKRK RR^{507} and ^{607}KKNRLRRK^{614} (Thakur et al., 1997)]. Chen et al. (1996b) reported that NLSs 503-508 and NLS 606-615, but not NLS 651-656, are crucial for nuclear localization as site directed mutagenesis of these sites result in cytoplasmic localization of BRCA1. Further studies have found that *BRCA1* interacts with the Importin-α subunit of the nuclear transport signal receptor. Thakur et al. (1997) also found that NLS 501-507 is critical for nuclear localization whereas deletion mutants lacking NLS 607-614 are nuclear. The discrepancy may be explained by the different mutations employed (site-directed mutagenesis versus deletion mutants). In any event, the characterization of the NLSs supports the idea that BRCA1 is a nuclear protein.

Thomas et al. (1996) have developed different antibodies (Table 1) and confirmed the nuclear localization of the 220 kDa *BRCA1* in both normal and cancer cell lines through immunofluorescence and biochemical fractionation. Moreover, Wilson et al. (1997) confirmed the nuclear staining using a panel of overexpressed epitope-tagged BRCA1 and biochemical fractionation of cells overexpressing BRCA1. Interestingly, they describe the major alternative splice variant *BRCA1* Δ11b (~ 110 kDa), that lacks the NLSs and localizes preferentially to the cytoplasm. This variant might be responsible for cytoplasmic staining in immunofluorescence since it conserves the N- and C-terminal epitopes against which the majority of the antibodies have been raised against. Even in the case of BRCA1 Δ11b variant, no evidence was found of staining in Golgi or endoplasmic reticulum (Wilson et al., 1997). Other groups raised additional antibodies (Table 1) used

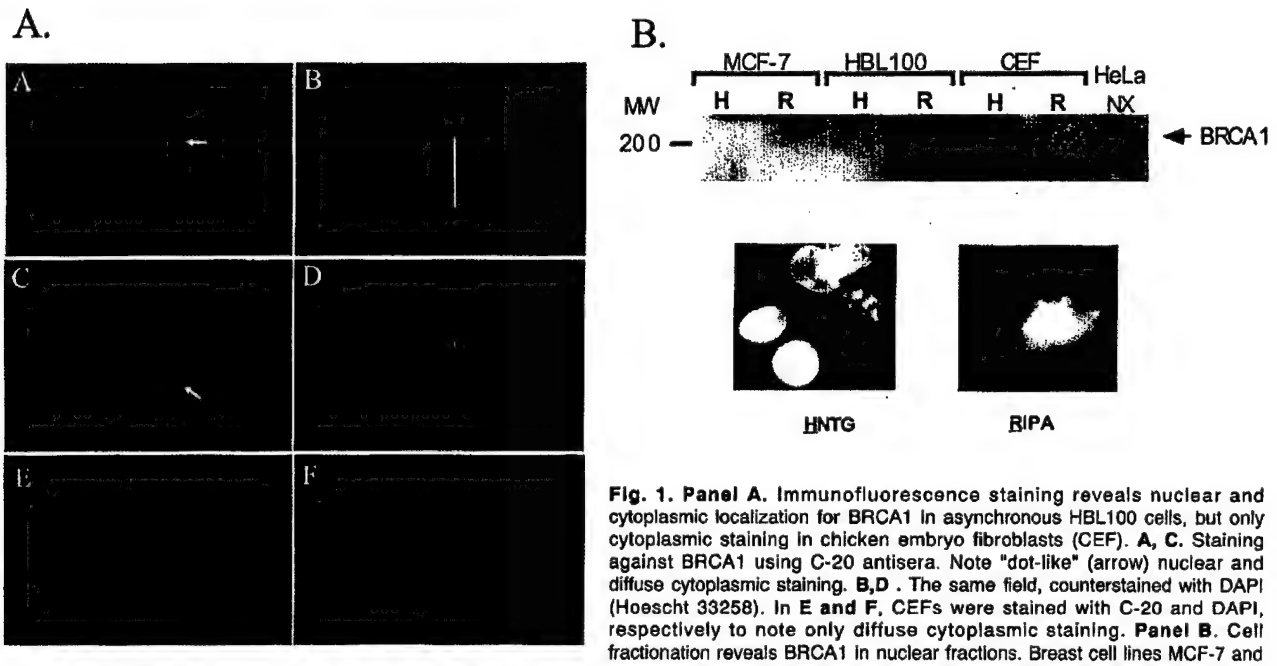

Fig. 1. Panel A. Immunofluorescence staining reveals nuclear and cytoplasmic localization for BRCA1 in asynchronous HBL100 cells, but only cytoplasmic staining in chicken embryo fibroblasts (CEF). **A, C.** Staining against BRCA1 using C-20 antisera. Note "dot-like" (arrow) nuclear and diffuse cytoplasmic staining. **B,D .** The same field, counterstained with DAPI (Hoescht 33258). In **E and F,** CEFs were stained with C-20 and DAPI, respectively to note only diffuse cytoplasmic staining. **Panel B.** Cell fractionation reveals BRCA1 in nuclear fractions. Breast cell lines MCF-7 and HBL100, and CEFs were lysed in 1% Triton-containing HNTG buffer, which is insufficient to lyse nuclear membranes or SDS-containing RIPA buffer, which readily lyses nuclear membranes (see lower DAPI panels). Detergent extracts from HNTG, RIPA, or high salt nuclear extracts (NX) were subsequently immunoprecipitated with C-20 antiserum and further immonoblotted with the same antiserum. BRCA1 was detected with HRP-conjugated secondary antibody.

for biochemical fractionation (Aprelikova et al., 1996; Thomas et al., 1996; Ruffner and Verma, 1997; Zhang et al., 1997) and immunoperoxidase staining (Rao et al., 1996) and have come to the conclusion that *BRCA1* is a nuclear protein with a molecular mass of 215-240 kDa.

Recently, Wilson et al. (1999) have undertaken an important task of comparing several different antibodies derived both from different laboratories and commercial sources in a variety of situations and consistently showed that BRCA1 is nuclear and attribute some early findings of cytoplasmic mislocalization to high concentrations of antibody used (see also Fig. 1). This study is the more comprehensive panel of antibodies tested so far and presents a compelling argument for a nuclear localization of BRCA1. More importantly, they present a series of biochemical fractionations using different antibodies to demonstrate nuclear localization. Table 1 in Wilson et al. (1999) summarizes the panel of antibodies, techniques and results obtained in parallel experiments. This table, used in conjunction with the Table 1 shown here, will be of special interest to the pathologist since it deals in detail with antibodies and conditions for immunohistochemistry in tissue blocks.

A function for BRCA1 in spindle checkpoints?

Hsu and White (1998) have presented interesting data suggesting the association of BRCA1 with the centrosome, more specifically with γ-tubulin during mitosis. Considering that the huge amounts of tubulin present in the cells are a potential cause of artifacts in immunoprecipitations, confirmation using other methods (in vitro binding assays or GST-fusion pull down) and other antibodies (besides MS110 and the problematic C-20) is needed before we can be certain. Importantly, it will be interesting to see if $BRCA1^{-/-}$ cell lines are prone to aberrant chromosome segregation caused by failure in spindle checkpoints.

A nuclear function?

Both genetics and cell biological data have led to the proposal that BRCA1 may be involved in DNA repair and in transcriptional regulation. BRCA1 contains a transcriptional activation domain localized to its C-terminus (Chapman and Verma, 1996; Monteiro et al., 1996). Interestingly, introduction of disease-predisposing mutations disrupted transcriptional activation (Chapman and Verma, 1996; Monteiro et al., 1996) while benign polymorphisms did not (Monteiro et al., 1997). In support of the proposed role in transcriptional regulation, BRCA1 has been found to be associated with the RNA polymerase II holoenzyme, through RNA helicase A (Scully et al., 1997a; Anderson et al., 1998) and to act as a coactivator for p53-mediated gene expression (Somasundaram et al., 1997; Ouchi et al., 1998; Zhang et al., 1998).

Co-localization studies by Scully et al. (1997b) have also uncovered an interaction of BRCA1 with Rad51, the homolog of bacterial RecA, suggesting a role for BRCA1 in DNA repair. In addition, DNA damage induces changes in BRCA1 subnuclear location and phosphorylation strengthening the idea that *BRCA1* is involved in DNA repair (Scully et al., 1997c; Thomas et al., 1997). Additional findings support the notion of BRCA1 may act in DNA repair, including the fact that blastocysts from $Brca1^{-/-}$ mice are more sensitive to DNA damage and tend to accumulate chromosomal abnormalities (Shen et al., 1998). Secondly, *BRCA1* has been found to be in a complex with BRCA2 (Chen et al., 1998), the product of the other major breast and ovarian cancer susceptibility gene that has also been shown to be involved in DNA repair (Connor et al., 1997; Sharan et al., 1997; Patel et al., 1998). Interestingly, a recent finding that BRCA1 is required for transcription-coupled repair in murine cells indicates that the above hypotheses for BRCA1 function are not mutually exclusive (Gowen et al., 1998).

BRCA1-Interacting proteins are nuclear

Screening methods, such as the yeast two-hybrid system, which are unbiased for proteins in any particular cellular compartment, have revealed the interaction of BRCA1 with two previously characterized proteins, c-Myc (Wang et al., 1998), a proto-oncogene that functions as a transcription factor and, CtIP a protein implicated in the CtBP pathway of transcriptional repression that was independently cloned by two laboratories (Wong et al., 1998; Yu et al., 1998). Both

Table 2. BRCA1-interacting proteins.

INTERACTING PROTEIN	BRCA1 BINDING SITE	LOCATION OF INTERACTING PROTEIN	FUNCTION OF INTERACTING PROTEIN	REFERENCE
BAP1	RING finger	nuclear	ubiquitin hydrolase	Jensen et al., 1998
BARD1	RING finger	nuclear	unknown, repair (?)	Wu et al., 1996
BRCA2	1314-1863	nuclear	transcription (?), repair	Chen at al., 1998
c-Myc	433-511	nuclear	transcription	Wang et al., 1998
CtiP	1651-1863	nuclear	transcription	Wong et al., 1998; Yu et al., 1998
γ-tubulin	n.d.	cytoplasm	cytoskeletal	Hsu et al., 1998
Importin-α	NLSs	nuclear/cytoplasm	nuclear import	Chen et al., 1996b
p53	224-500	nuclear	transcription	Ouchi et al., 1998; Zhang et al., 1998
RAD51	758-1064	nuclear	repair	Scully et al., 1997b
RNA helicase A	1650-1800	nuclear	transcriptional	Anderson et al., 1998

proteins are nuclear and their interaction with BRCA1 is consistent with the proposed role of BRCA1 in transcription.

Two previously unknown proteins were also found, BARD1, a protein of unknown function containing a RING finger and two BRCT domains (Wu et al., 1996) and BAP-1 a protein with ubiquitin hydrolase activity (Jensen et al., 1998). With exception of γ-tubulin, all the proteins found to interact in vivo with *BRCA1* are nuclear or participate in nuclear import (Table 2).

Conclusion

The controversy about BRCA1 is a cautionary tale for cell biologists as well as pathologists and illustrates the difficulty to unambiguously assign a location to a protein of unknown function. There is an overwhelming body of direct evidence that points to a nuclear localization and function for BRCA1 in normal and cancer cells. Similarly, there is an increasing amount of indirect evidence, both from interacting proteins and from functional studies of BRCA1, that support the data for the nuclear localization for BRCA1. It is too soon to evaluate the functional significance and relevance of the interacting proteins identified in several screening approaches. One way to assess the significance of the interaction is to show that interaction is disrupted by disease-causing mutations, which has been shown in some cases. In any case, results from interaction experiments seem to support the notion that BRCA1 is involved in repair and in transcriptional regulation.

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BRCA1 can stimulate gene transcription by a unique mechanism

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Most familial breast and ovarian cancers have been linked to mutations in the *BRCA1* gene. BRCA1 has been shown to affect gene transcription but how it does so remains elusive. Here we show that BRCA1 can stimulate transcription without the requirement for a DNA-tethering function in mammalian and yeast cells. Furthermore, the BRCA1 C-terminal region can stimulate transcription of the p53-responsive promoter, *MDM2*. Unlike most enhancer-specific activators, non-tethered BRCA1 does not require a functional TATA element to stimulate transcription. Our results suggest that BRCA1 can enhance transcription by an function additional to recruiting the transcriptional machinery to a targeted gene.

INTRODUCTION

BRCA1 mutations are thought to account for ~45% of families with high breast cancer risk and >80% of families with high risk of early-onset breast and ovarian cancer (Easton *et al.*, 1993). The human *BRCA1* gene encodes a 1863 amino acid nuclear protein that has been implicated in DNA repair and transcription activation (see Monteiro, 2000; Welcsh *et al.*, 2000). A role for BRCA1 in gene transcription has been proposed mainly for the following reasons: (i) the C-terminal portion of BRCA1, which bears an excess of negatively charged residues, can activate transcription of a target gene when attached to a DNA-binding domain (DBD) *in vivo* (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Anderson *et al.*, 1998) and *in vitro* using a highly purified system (Haile and Parvin, 1999); (ii) it associates with a form of RNA polymerase II holoenzyme (Scully *et al.*, 1997); and (iii) it modulates the activity of certain transcriptional activators (Somasundaram *et al.*, 1997; Ouchi *et al.*, 1998, 2000; Wang *et al.*, 1998). It is noteworthy that the above mentioned work implies BRCA1 as a physiological regulator of p53-dependent genes such as *p21WAF* and *MDM2*.

Here we show that BRCA1 can stimulate gene transcription of a variety of reporter gene constructs without the requirement for a specific DBD in human and in yeast cells both *in vivo* and *in vitro*. We further show that the BRCA1 C-terminal region can increase transcription of a reporter gene independently of a functional TATA element.

RESULTS

BRCA1 stimulates transcription without requiring a specific DBD in human cells

While studying the effect of the BRCA1 C-terminal region on gene transcription, we noticed that expression of a protein fragment (aa 1528–1863) could efficiently stimulate gene transcription in transiently transfected human cells. We thus hypothesized that perhaps the activation elicited by a DNA-tethered BRCA1 chimera (e.g. Gal4–BRCA1) might not be dependent on a heterologous DBD. To test this hypothesis, we transfected human HCC1937 *BRCA1*$^{-/-}$ breast cancer cells (Tomlinson *et al.*, 1998) with a vector overexpressing Gal4–BRCA1(aa 1528–1863) or the BRCA1 fragment without the Gal4 DBD. As previously reported (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Anderson *et al.*, 1998), the Gal4–BRCA1 fusion activated transcription from a reporter template bearing four Gal4-binding sites upstream of the c-*fos* minimal promoter fused to the luciferase gene (Figure 1A). Interestingly, the BRCA1 fragment, without a Gal4 DBD, was also able to enhance the level of transcription several-fold in a dose-dependent fashion.

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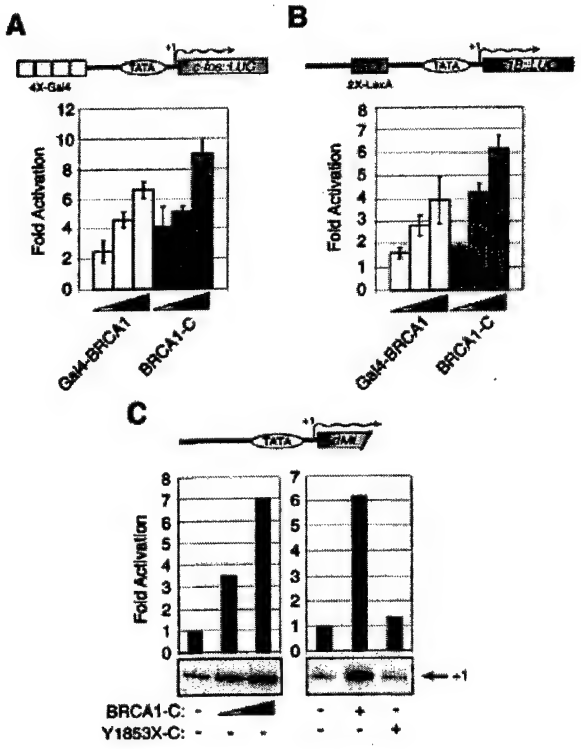

Fig. 1. BRCA1 does not require specific DNA binding to enhance gene transcription. **(A)** Activation by Gal4–BRCA1(1528–1863) and BRCA1(1528–1863) at a reporter template bearing Gal4 sites in HCC1937 cells. Transfected cells were assayed for luciferase activity using a reporter template (0.1 μg) bearing four Gal4-binding sites upstream of the c-*fos* TATA element. Increasing amounts (0.5 and 1.0 μg DNA) of either Gal4–BRCA1(1528–1863) or BRCA1-C(1528–1863) constructs were co-transfected along with the reporter. Activation for BRCA1 and Gal4–BRCA1 is represented as fold increase over activity obtained with the pcDNA3 vector and the Gal4 DBD, respectively. **(B)** Activation by Gal4–BRCA1 and BRCA1-C at a reporter template lacking Gal4 sites. Transfected HCC1937 cells were assayed for luciferase activity using 0.1 μg of a reporter bearing two LexA sites upstream of the *E1B* TATA element. The BRCA1 plasmid constructs were transiently transfected as in (A) and activation is represented as the fold increase over activity obtained with the pcDNA3 vector for both constructs. **(C)** BRCA1 can stimulate gene transcription *in vitro*. *In vitro* transcription reactions were carried out with a HeLa nuclear extract and an *AdML* template. Primer extensions were carried out to measure the extent of activation. Recombinant BRCA1(1528–1863) was added at 100 and 400 ng (left panel, lanes 2 and 3, respectively). The right panel shows that a recombinant Y1853X-bearing mutant (200 ng, lane 3) does not significantly stimulate transcription as compared with the wild-type protein fragment (200 ng, lane 2).

To determine whether the effect could be specific to the promoter context of the template, we made use of a promoter template bearing two LexA-binding sites in place of the Gal4 sites, and the *E1B* core promoter in place of the c-*fos* promoter. The results show that, even though both the Gal4–BRCA1 fusion and BRCA1 are not expected to bind the promoter template, they are both able to stimulate transcription when overexpressed in the cell (Figure 1B). These results would be expected for an activator that does not require DNA binding to exert its function.

Next, we tested whether the BRCA1 C-terminal fragment could activate transcription *in vitro* without the requirement for a DBD. Using a HeLa nuclear extract, we tested the activation potential of recombinant BRCA1(1528–1863) *in vitro* using a DNA template bearing the *AdML* core promoter. We found that the recombinant BRCA1 fragment could stimulate transcription up to 7-fold (Figure 1C), a result consistent with those of our transient transfection experiments. A similar BRCA1 fragment bearing the cancer-associated Y1853X mutation, which results in deletion of the last 11 amino acids (Friedman *et al.*, 1994), had no significant stimulatory effect on transcription (Figure 1C, right panel, lane 3).

Transcription stimulation of *MDM2* by BRCA1 derivatives

Previous reports have suggested that p53-responsive genes were targets of BRCA1-mediated transcription enhancement. Moreover, p53 has been shown to interact physically with residues 224–500 of BRCA1 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). Although physical interaction between these two molecules surely contributes in recruiting BRCA1 to p53-responsive genes, it is conceivable that proper overexpression of the BRCA1 activation-effector region would also result in stimulation of the target gene. We thus wanted to test whether the BRCA1 C-terminal region was sufficient, as compared with the full-length molecule, to stimulate transcription of an *MDM2-luciferase* reporter template (Ouchi *et al.*, 1998). To test this, we transfected HCC1937 cells with vectors expressing C-terminal and full-length BRCA1 derivatives (wild-type and Y1853X mutants), and luciferase activity was analyzed. The results of Figure 2 show that the C-terminal BRCA1 fragment, as well as full-length BRCA1, can efficiently induce transcription elicited at the *MDM2* promoter while the Y1853X mutants did not stimulate the reporter as efficiently. These results suggest that overexpression of BRCA1 can bypass the requirement for a p53–BRCA1 interaction in order to stimulate a responsive gene.

The BRCA1 C-terminal region stimulates transcription of a TATA-mutated promoter

In an effort to discriminate further the mechanism of action of BRCA1, as compared with enhancer-binding activators, we set out to test whether BRCA1 required a functional TATA element to stimulate transcription. Hence, we determined the ability of BRCA1 to activate transcription at a reporter template that has a mutation in the TATA element (TGTA) (Figure 3A). This mutation prevents or severely reduces TBP binding to the core promoter, nearly abolishing the ability of a classical activator to activate transcription in certain contexts (Bryant *et al.*, 1996). Thus, the reporter templates used in this experiment have either a wild-type c-*fos* TATA or a mutated TGTA element both with Gal4-binding sites upstream and the *luciferase* reporter gene downstream of the transcription initiation site (Bryant *et al.*, 1996). While the acidic activators Gal4–VP16 and Gal4–E2F1 are able to activate transcription efficiently at the TATA template, both activators are severely crippled in their ability to stimulate transcription at the TGTA template. Surprisingly, BRCA1(1528–1863) was able to stimulate transcription even more efficiently at the TGTA template as compared with the TATA template (Figure

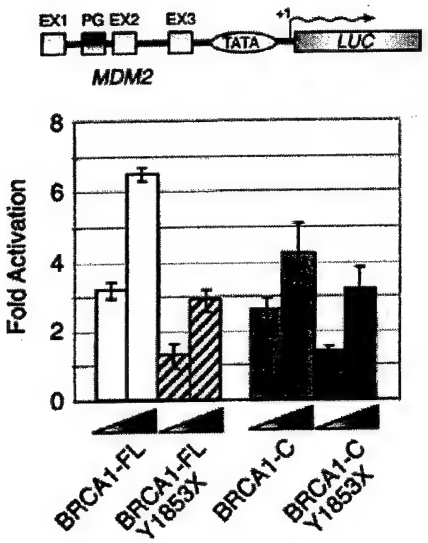

Fig. 2. Transcription stimulation of the *MDM2* promoter by BRCA1 derivatives. HCC1937 cells were transfected with various BRCA1 derivatives (using either 0.5 or 1.0 μg of DNA) and 25 ng of an *MDM2-luciferase* template (Ouchi *et al.*, 1998). Exons 1–3 (EX1–EX3) and p53-responsive elements (PG) are indicated. The vector control denotes pcDNA3; BRCA1-C denotes BRCA1(1528–1863); BRCA1-C Y1853X is the BRCA1 C-terminal region with the Y1853X mutation; and BRCA1-FL are the full-length molecules. Activation was measured as in Figure 1A.

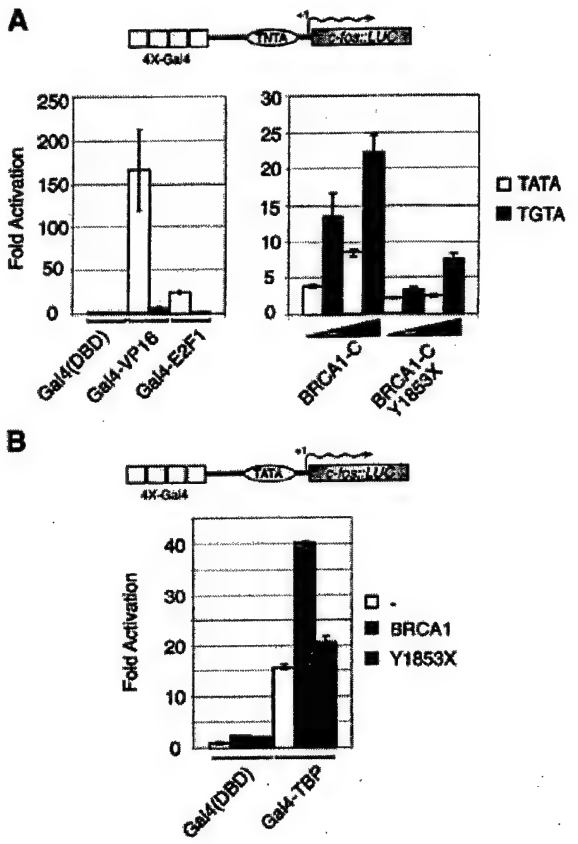

Fig. 3. BRCA1 stimulates transcription independently of TBP binding. **(A)** BRCA1 can efficiently stimulate transcription in the absence of a functional TATA element. HCC1937 cells were transfected with either a reporter template bearing a functional TATA element or one with a mutated TATA (TGTA) otherwise identical. The left part of the figure measures the ability of the Gal4 DBD, Gal4–VP16 and Gal4–E2F1 to activate transcription at both templates. The right part of the figure measures the ability of various BRCA1 derivatives (using either 0.5 or 1.0 μg of DNA) to stimulate both reporters. The vector control denotes pcDNA3; BRCA1-C denotes BRCA1(1528–1863); BRCA1-C Y1853X is the BRCA1 C-terminal region with the Y1853X mutation. Activation was measured as in Figure 1A and B. **(B)** The BRCA1 C-terminal region stimulates activation elicited by Gal4–TBP. Transiently transfected HEK-293 cells were assayed for luciferase activity using the reporter template of Figure 1A. Either the Gal4 DBD (1–147) (0.2 μg) or Gal4–TBP (0.2 μg) was used in the experiment with or without 0.4 μg of either BRCA1-C derivative (wild-type or Y1853X mutant).

3A, right panel). Introduction of a Y1853X mutation markedly impaired stimulation of transcription.

We next wanted to test whether the BRCA1 C-terminal region could further enhance transcription of a gene activated by artificially recruited TBP. Figure 2B shows that TBP, when fused to the Gal4 DBD, can activate transcription some 15-fold in human HEK-293 cells. When BRCA1(1528–1863) is transfected along with Gal4–TBP, we see an ~3-fold increase in activation by Gal4–TBP. Transfection of the Y1853X BRCA1 mutant did not enhance transcription of Gal4–TBP nearly as efficiently as its wild-type counterpart. These results suggest that BRCA1 can stimulate transcription above levels elicited by artificial recruitment of TBP to the TATA box. We cannot, however, exclude the possibility that BRCA1 further enhances TBP binding to the promoter beyond the recruiting effect of the Gal4 DBD.

BRCA1 stimulates transcription without requiring a specific DBD in yeast cells

To determine whether the effect of BRCA1 on transcription was limited to mammalian cells, we performed experiments in yeast. Figure 4A shows that, when expressed in yeast cells, full-length BRCA1 is able to stimulate transcription (bar 2) of a *GAL1::lacZ* reporter template efficiently. Expression of the Y1853X mutant (Figure 4A, bar 4) in yeast failed to activate transcription, while the C61G mutant (bar 3), also a clinically relevant mutation but located at the N-terminus of BRCA1 (Friedman *et al.*, 1994), could elicit levels of activation comparable to the wild-type protein. The N-terminal region of BRCA1 seems dispensable for this function, confirming our data from mammalian cells where the C-terminal alone is sufficient for activation. Figure 4B shows that recombinant BRCA1(1528–1863) can stimulate transcription of the *GAL1* promoter *in vitro* using a yeast nuclear extract preparation (Wu *et al.*, 1995). The figure also shows that, as in Figure 1C, BRCA1 can stimulate transcription in a dose-dependent fashion. The Y1853X mutant had no stimulatory effect (Figure 4B, right panel, lane 3).

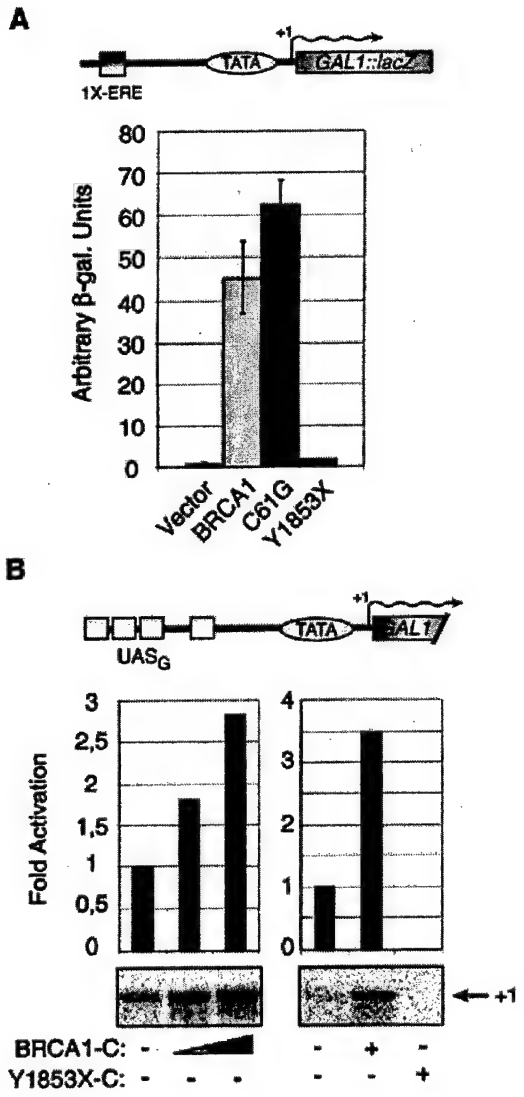

Fig. 4. BRCA1 can stimulate gene transcription in yeast without the requirement for a DBD. **(A)** *In vivo*. The ability of BRCA1(1–1863) to stimulate transcription in yeast without a DBD was assessed in a yeast strain harboring a *lacZ* reporter template with an ER binding site upstream of the *GAL1* TATA. Activation was measured as arbitrary β-galactosidase units. **(B)** *In vitro*. *In vitro* transcription reactions were carried out with a yeast nuclear extract and *GAL1* DNA template. Primer extensions were carried out as in Figure 1C. Recombinant BRCA1(1528–1863) was added at 200 and 400 ng (lanes 2 and 3, respectively). The right panel depicts a Y1853X control (200 ng, lane 3) along with BRCA1 (200 ng, lane 2) as in Figure 1C.

DISCUSSION

Our results show that the BRCA1 C-terminal region has the surprising ability to activate transcription without the need for a DNA-binding function and, in so doing, exploits a mechanism different from the one typified by many enhancer-binding activators. Importantly, these activities were crippled upon introduction of a cancer-associated mutation, Y1853X, which destabilizes the BRCT region leaving one of the two BRCT motifs intact. This intact BRCT could perhaps explain the transcriptional activity of that mutant when it is well overexpressed in some experiments. We have also shown that the C-terminal region of BRCA1, as well as the full-length molecule, could stimulate transcription of a p53-responsive promoter. The experiments described here rely on overexpression of BRCA1 derivatives and could imply a global effect on gene transcription, considering that BRCA1 can activate genes with a variety of promoter contexts. However, we propose that our experimental conditions represent increased local concentrations of BRCA1 under physiological conditions, a condition that we believe bypasses specific targeting of BRCA1 to our reporters, a situation well exemplified by our experiment with the *MDM2* reporter.

Our results showing that BRCA1 does not require a functional TATA element to stimulate transcription could imply, for example, that either BRCA1 stabilizes binding of the transcription machinery (e.g. TFIID) to the mutated promoter or, alternatively, BRCA1 could act independently of TBP binding to DNA to stimulate transcription. Recently, oligonucleotide array-based expression profiling experiments have revealed that BRCA1 expression could efficiently induce transcription of the DNA damage-responsive gene *GADD45* in a p53-independent fashion (Harkin *et al.*, 1999). Interestingly, examination of the *GADD45* proximal promoter region did not reveal the presence of any consensus TATA element (not shown). Thus, the fact that BRCA1 does not require a functional TATA element to stimulate transcription would be relevant in this particular case.

In prokaryotes, there are at least three examples of activators that do not require sequence-specific DNA-binding activity in order to stimulate transcription: (i) DNA-tracking proteins exemplified by the phage T4 Gp54 one-dimensional sliding clamp; (ii) the phage N4 single-stranded binding protein (N4SSB), which interacts with the β′ subunit of RNA polymerase and activates σ^{70}-type promoters; and (iii) σ^{54}-specific activators, which, even if being enhancer-binding proteins, can activate transcription at high concentrations without being DNA tethered (see Ptashne and Gann, 1997; Hochschild and Dove, 1998). It is conceivable that BRCA1 could stimulate transcription by a mechanism similar to any of the bacterial examples listed above. Recent experiments carried out with the TATA-binding protein-interacting protein TIP120 and ABT1 have shown that they too can enhance transcription without requiring a DNA-tethering function (Makino *et al.*, 1999; Oda *et al.*, 2000). Interestingly, our results could explain why BRCA1 activates the *p21WAF1* gene independently of p53 (Somasundaram *et al.*, 1997; Ouchi *et al.*, 1998) and also IFN-γ target genes in the absence of interferon stimulation (Ouchi *et al.*, 2000).

We consider that the mechanism by which BRCA1 stimulates transcription could constitute an advantage in cases where the transcriptional machinery might be paused, for example, at sites of DNA damage in a particular gene, located at a distance from its enhancer sequences. In the latter case, BRCA1 would be able to counteract a transcriptional pause without any interaction(s) with the upstream enhancer. Consistent with this idea is a recent report which suggests that BRCA1 BRCT domains can bind DNA

strand breaks and termini (Yamane and Tsuruo, 1999). Thus, it is conceivable that BRCA1 could recognize damaged DNA at paused transcription sites, and in so doing, increase favorable interactions with the transcriptional machinery thereby further enhancing transcription and perhaps, the transcription-coupled DNA repair process itself. In an alternative mode not mutually exclusive with the previous one, BRCA1 could be targeted to specific genes by interaction with enhancer-binding activators and in so doing, further enhance the transcription process.

METHODS

Transient transfections. Human HCC1937 and HEK-293 cells were used for the transfection experiments and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated in six-well tissue culture plates for 24 h before transfection at a density of $1–2 \times 10^5$ cells/well. Cells were then transfected using either Fugene 6 or Lipofectamine 2000 reagents. After 18–24 h, cells were processed for luciferase assays. Transfection efficiencies, when appropriate, were normalized using the pβgal plasmid; in some experiments, we noticed that BRCA1 increased the activity of our *lacZ* internal control, thus, in many experiments we did not normalize our transfection efficiencies; nonetheless we always measured *lacZ* values to make sure no gross deviations would be observed. Details on plasmid constructions are available upon request.

Recombinant BRCA1 derivatives. Recombinant BRCA1(1528–1863) was expressed in *Escherichia coli* using the pET30a expression vector. The recombinant protein was first chromatographed on Ni-NTA agarose and then further fractionated on an FPLC mono-Q ion exchange column. The Y1853X mutant fragment was also expressed in *E. coli*, affinity purified on Ni-NTA agarose and then subjected to chromatography on a Superdex 200 HR 10/30 column.

Nuclear extracts and *in vitro* transcription. HeLa and HCC1937 nuclear extract preparation and transcription reactions were as previously described (Shapiro *et al.*, 1988). Yeast nuclear extract preparation and transcription reactions were as described in Wu *et al.* (1995)) and supplemented with recombinant TBP and TFIIE. For human *in vitro* transcription experiments, the pGML4 template was used at 100 ng/reaction; pGML4 bears the *AdML* promoter without any enhancer sequences. For yeast *in vitro* transcription experiments, the pGDC01 template was used at 100 ng/reaction; pGDC01 bears the *GAL1* promoter with its native UAS_G. All data were quantified by phosphoimaging.

Yeast manipulations. The yeast strain TGY14 (*MATa, ura3-251-373-328, leu2, pep4.3*) was used and contained a *GAL1::lacZ* reporter gene inserted downstream of an ERE. Competent yeast cells were obtained using the yeast transformation system (Clontech) based on lithium acetate, and cells were transformed according to the manufacturer's instructions. Activity of the reporter was measured by liquid β-galactosidase assays.

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Functional Assay for BRCA1: Mutagenesis of the COOH-Terminal Region Reveals Critical Residues for Transcription Activation[1]

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ABSTRACT

The breast and ovarian cancer susceptibility gene product BRCA1 is a tumor suppressor, but its precise biochemical function remains unknown. The BRCA1 COOH terminus acts as a transcription activation domain, and germ-line cancer- predisposing mutations in this region abolish transcription activation, whereas benign polymorphisms do not. These results raise the possibility that loss of transcription activation by BRCA1 is crucial for oncogenesis. Therefore, identification of residues involved in transcription activation by BRCA1 will help understand why particular germ-line missense mutations are deleterious and may provide more reliable presymptomatic risk assessment.

The BRCA1 COOH terminus (amino acids 1560–1863) consists of two BRCTs preceded by a region likely to be nonglobular. We combined site-directed and random mutagenesis, followed by a functional transcription assay in yeast: (*a*) error-prone PCR-induced random mutagenesis generated eight unique missense mutations causing loss of function, six of which targeted hydrophobic residues conserved in canine, mouse, rat, and human BRCA1; (*b*) random insertion of a variable pentapeptide cassette generated 21 insertion mutants. All pentapeptide insertions NH_2-terminal to the BRCTs retained wild-type activity, whereas insertions in the BRCTs were, with few exceptions, deleterious; and (*c*) site-directed mutagenesis was used to characterize five known germ-line mutations and to perform deletion analysis of the COOH terminus. Deletion analysis revealed that the integrity of the most COOH-terminal hydrophobic cluster (I1855, L1854, and Y1853) is necessary for activity. We conclude that the integrity of the BRCT domains is crucial for transcription activation and that hydrophobic residues may be important for BRCT function. Therefore, the yeast-based assay for transcription activation can be used successfully to provide tools for structure-function analysis of BRCA1 and may form the basis of a BRCA1 functional assay.

INTRODUCTION

Individuals carrying mutations in the *BRCA1* gene have an increased risk of developing breast and ovarian cancer (1). Mutations in *BRCA1* alone account for ~45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (2). After an extensive search, *BRCA1* was mapped to the long arm of chromosome 17 by linkage analysis (3) and was cloned by positional cloning techniques (4). Human *BRCA1* codes for a 1863-amino acid protein with no detectable similarity to known proteins, with the exception of a zinc-binding RING finger domain located in the NH_2-terminal region (4), and two BRCT[4] domains found in a variety of proteins involved in cell cycle control and DNA repair (5–7).

Recent evidence points to the involvement of BRCA1 in two basic cellular processes: DNA repair and transcriptional regulation. BRCA1 is present in a complex containing Rad51 (8) and BRCA2 (9), and DNA damage may control BRCA1 phosphorylation and subnuclear location (10, 11), strongly suggesting its involvement in the maintenance of genome integrity. Additional evidence for the role of BRCA1 in maintenance of genome integrity is provided by targeted disruption of *Brca1* in the mouse. Mouse embryos lacking *Brca1* are hypersensitive to γ-irradiation, and cells display numerical and structural chromosomal aberrations (12).

We and others have shown that the BRCA1 COOH terminus has the ability to activate transcription in mammalian and yeast cells and that the introduction of germ-line disease-associated mutations, but not benign polymorphisms, abolishes this activity (13–15). BRCA1 can be copurified with the RNA polymerase II holoenzyme, supporting the idea that BRCA1 is involved in transcription regulation (16, 17). In addition, BRCA1 causes cell cycle arrest via transactivation of $p21^{WAF1/CIP1}$ (18) and regulates p53-dependent gene expression, acting as a coactivator for p53 (19, 20). In all of these studies, the COOH-terminal region was necessary for activity. It is still not clear whether BRCA1 is a multifunctional protein with repair and transcription regulation functions or whether the role of BRCA1 in repair is mediated through transcription activation. In either case, these functions are not necessarily mutually exclusive.

The dearth of knowledge concerning the precise biochemical function of BRCA1 is a major hurdle in developing a functional test to provide reliable presymptomatic assessment of risk for breast and ovarian cancer. The available data derived from linkage analysis indicate that all mutations that cause premature termination (even relatively subtle mutations such as the deletion of 11 amino acids from the COOH terminus) will confer high risk (21). However, a considerable number of mutations result in amino acid substitutions that, in the absence of extensive population-based studies or a functional assay, do not allow assessment of risk. Two related yeast-based assays designed to characterize mutations in the BRCA1 COOH terminal region have generated results that provide an excellent correlation with genetic linkage analysis (13, 14, 22). This led us to propose the general use of a yeast-based assay to provide functional information and a more reliable risk assessment (23).

In this report, we use site-directed and random mutagenesis to generate mutations in the BRCA1 COOH terminal region that disrupt transcription activation with the intention of both defining critical residues for BRCA1 function and deriving general rules to predict the impact of a particular mutation.

MATERIALS AND METHODS

Yeast Strains. Three *Saccharomyces cerevisiae* strains were used in this study: HF7c [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4–542, gal80–538, LYS::GAL1-HIS3, URA3::(GAL4 17mers)$_3$-CYC1-lacZ*]; SFY526 [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3,112,* can^r, *gal4–542, gal80–538, URA3::GAL1-lacZ*]

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[4] The abbreviations used are: BRCT, BRCA1 COOH terminal domain; DBD, DNA binding domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MTD, minimal transactivation domain.

(24); and EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] (25). HF7c has an *HIS3* reporter gene under the control of the *GAL1* upstream activating sequence, responsive to GAL4 transcription activation. The vectors used for expression confer growth in the absence of tryptophan (see below). The SFY526 strain has a *lacZ* reporter under the control of *GAL1* upstream activating sequence and was transformed with the GAL4 DBD fusion. EGY48 cells were cotransformed with the LexA fusion vectors and plasmid reporters of *lacZ* under the control of LexA operators (see below). If the fusion proteins activate transcription, EGY48 and SFY526 yeast transformants will produce β-galactosidase, and HF7c transformants will grow in medium lacking histidine.

Yeast Expression Constructs. The GAL4 DBD fusion of the wild-type human BRCA1 COOH terminal region (amino acids 1560–1863) was described previously (13). Alternatively, this fragment was subcloned into the yeast expression vector pLex9 (25) in-frame with the DBD of LexA. Both plasmids have *TRP1* as a selectable marker, allowing growth in the absence of tryptophan. We noticed that our previously described BRCA1 (amino acids 1560–1863) construct (13) was made with a 3′ primer lacking a termination codon. This introduces 16 exogenous amino acids to the COOH-terminal region of BRCA1. We have corrected this by using primer 24ENDT (5′-GCGGATCCTCAGTAGTGGCTGTGGGGGAT-3′). We compared both constructs and ascertained that qualitatively and quantitatively, they have the same activity (not shown).

BRCA1 deletion mutants were generated by PCR on a BRCA1 (amino acids 1560–1863) context using pcBRCA1–385 (a gift from Michael Erdos, National Human Genome Research Institute) as a template and the following primers: H1860X (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S970074, 5′-GCGGATCCTCAGGGGATCTGGGG-3′); P1856X (S9503101, S970073, 5′-GCGGATCCTCATATCAGGTAGGTGTCC-3′); I1855X (S9503101, 1855STOP, 5′-GCGGATCCTCACAGGTAGGTGTCC-3′); and L1854X (S9503101, 1854STOP, 5′-GCGGATCCTCAGTAGGTGTCCAGC-3′). Mutant Y1853X corresponds to a germ-line mutation and has been described previously (13). The constructs were sequenced to verify the mutations. The PCR products were digested with *Eco*RI and *Bam*HI and subcloned into similarly digested pGBT9 vectors. Alternatively, the PCR fragments were subcloned into a vector, pAS2–1 (Clontech), with higher expression levels. Introduction of additional mutations was made using the Quick-Change method. Briefly, a pair of primers encoding each mutation flanked by homologous sequence on each side was added to the wild-type plasmid pLex9 BRCA1 (amino acids 1560–1863) prepared in a methylation-competent strain. The plasmid was amplified using *Pfu* polymerase (one cycle at: 96°C for 30 s; 12 cycles at: 96°C for 30 s; 50°C for 1 min; and 68°C for 12 min), and *Dpn*I was added at the end of the reaction to digest the parental plasmid. The mixture was then transformed into bacteria. The following oligonucleotide primers were used: T1561I (T1561IF, 5′-CTGGAATTCGAGGGAATCCCTTACCTCGAGTCTGG-3′; T1561IR, 5′-CCAGACTCGAGGTAAGGGATTCCCTCGAATTCCAG-3′); L1564P (L1564PF, 5′-GGGTACCCCTTACCCGGAATCTGGAATCAG-3′;L1564PR,5′-CTGATTCCAGATTCCGGGTAAGGGGTACCCTC-3′); D1733G (D1733GF, 5′-GAAAAATGCTCAATGAGCATGGTTTTGAAGTCCGCGGAG-3′; D1733GR, 5′-CTCCGCGGACTTCAAAACCATGCTCATTCAGCATTTTTC-3′); G1738E (G1738EF, 5′-GAGCATGATTTTGAAGTCAGAGAAGATGTGGTTAACGGAAG-3′; G1738ER, 5′-CTTCCGTTAACCACATCTTCTCTGACTTCAAAATCATGCTC-3′); P1806A (P1806AF, 5′-GGTACCGGTGTCCACGCAATTGTGGTTGTGCAGC-3′; and P1806AR, 5′-GCTGCACAACCACAATTGCGTGGACACCGGTACC-3′).

Yeast Plasmid Reporters. Plasmid pSH18-34 (25), a kind gift of Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA), was used as a reporter in the LexA fusion assays. This vector has *lacZ* under the control of eight LexA operators, conferring low stringency of gene expression (26).

Yeast Transformation. Competent yeast cells were obtained using the yeast transformation system (Clontech) based on lithium acetate, and cells were transformed according to the manufacturer's instructions.

Filter β-Galactosidase Assay. SFY526 and EGY48 transformants (several clones for each construct) were streaked on a filter overlaid on solid medium lacking tryptophan (SFY526) or tryptophan and uracil (EGY48) and allowed to grow overnight. Cells growing on the filter were lysed by freeze/thawing in liquid nitrogen, and each filter was incubated in 2.5 ml of Z buffer (16.1 g/liter $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, and 0.246 g/l $MgSO_4{\cdot}7H_2O$, pH 7.2) containing 40 μl of X-gal solution (20 mg/ml of X-gal in *N,N*-dimethylformamide) at 30°C for up to 16 h.

Liquid β-Galactosidase Assay. Liquid assays were performed as described previously (27). At least three separate transformants were assayed, and each was performed at least in duplicate.

Growth Curves. HF7c transformants (several clones) containing different pGBT9 or pAS2 constructs were grown overnight in synthetic medium plus 2% dextrose (SD medium) lacking tryptophan. The saturated cultures were used to inoculate fresh medium lacking tryptophan or tryptophan and histidine to an initial A_{600} of 0.0002. Cultures were grown at 30°C in the shaker, and the absorbance was measured at different time intervals starting at 12 h, then every 4 h up to 36 h after inoculation.

Plasmid Recovery from Yeast Cells. EGY48 transformants were grown to saturation in liquid medium lacking uracil (but in the presence of tryptophan). Cells were harvested and treated with yeast lysis solution [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA], phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 g of acid-washed beads. The sample was vortexed for 2 min and centrifuged, and the supernatant precipitated with one-tenth volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of ethanol. Alternatively, plasmid rescue was performed as suggested by Strathern and Higgins (28).

Screening in X-gal Plates. To allow direct screening of the clones with loss of activity, EGY48 cells transformed with the mutagenized cDNAs were plated on X-gal-containing plates: 2% galactose, 1% raffinose, 80 mg/l X-gal, and 1× BU salts (1 liter of 10× BU salts: 70 g $Na_2HPO_4{\cdot}0.7H_2O$, 30 g NaH_2PO_4).

Error-prone PCR Mutagenesis. A 60-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using Taq polymerase and p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). The PCR product was gel purified and cotransformed in an equimolar ratio with a *Nco*I-linearized wild-type pLex9 BRCA1 (amino acids 1560–1863) plasmid and pSH18-34. After transformation, cells were plated on X-gal plates and incubated for 5 days. Eighty-one white and four control blue clones were recovered and restreaked on master plates. White clones were screened again on a filter assay, and the 62 clones that were consistently white were analyzed further. Plasmid DNA was recovered from the yeast cells and transformed into *Escherichia coli*. Miniprep DNAs from each of two bacterial transformants from the 62 candidates were retransformed into yeast cells and tested again for β-galactosidase production. The BRCA1 inserts in plasmid DNAs generating white clones were subjected to direct sequencing using dye terminators.

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a technique whereby 5-amino acid insertions are introduced at random in a target protein (29). Briefly, an *E. coli* donor strain containing the target plasmid and pHT385, a conjugative delivery vector for transposon Tn*4430*, is mated with a plasmid-free *E. coli* recipient strain. By plating the mating mix simultaneously on antibiotics selecting for the recipient, the target plasmid, and Tn*4430*, transconjugants containing pHT385::target plasmid cointegrates are isolated. This cointegrate resolves rapidly *in vivo*, regenerating pHT385 and the target plasmid into which a copy of Tn*4430* has been inserted. Tn*4430* contains *Kpn*I restriction enzyme sites located 5-bp from both ends of the transposon and duplicates 5-bp of target site sequence during transposition. By digesting the target plasmid::Tn*4430* hybrid with *Kpn*I and religating the digested DNA, the bulk of the transposon is deleted to generate a target plasmid derivative containing a 15-bp insertion. If the insertion is in a protein-encoding sequence, this will result in a 5-amino acid insertion in the target protein.

Tn*4430* insertions in the COOH-terminal region of BRCA1 were identified either by genetic or physical means. In the former case, 30 separate matings were performed as detailed previously (30) using appropriate antibiotic selections and in which the target plasmid was pLex9 containing the BRCA1 COOH terminal region fused to LexA DBD. Transconjugant colonies were harvested by washing from the mating plates, and plasmid DNA was isolated from the pooled colonies. The plasmid preparations were pooled further and transformed into *Saccharomyces cerevisiae* EGY48 harboring the pSH18-34 reporter plasmid. Transformants were tested for transcription activation by replica-plating to plates containing X-gal. Plasmid DNA was recovered from white colonies and transformed into *E. coli* XL1-Blue selecting on X-gal-

Table 1 *Missense mutations leading to loss of function (PCR-mediated mutagenesis screen)*

Exon	Mutation	Dog[a]	Mouse[b]	Rat[c]	Nucleotide[d]	Base change	Comments and probable secondary structure elements[e]
16	M1652K	M	M	M	5074	T to A	Residue mutated in germ line (M1652T, M1652I).
18	K1702E	K	K	K	5223	A to G	α-Helix 2 of BRCT-N.
18	Y1703H	Y	Y	Y	5226	T to C	α-Helix 2 of BRCT-N.
18	L1705P	L	L	L	5233	T to C	Found in two independent clones. Located just after α-helix 2 of BRCT-N.
21	F1761S	F	F	F	5401	T to C	BRCT-N/BRCT-C interval.
21	F1761I	F	F	F	5400	T to A	BRCT-N/BRCT-C interval.
22	L1780P	L	L	L	5458	T to C	α-Helix 1 of BRCT-C. Hydrophobic residue conserved in BRCT superfamily. Mediates interaction between α-helix 1 and α-helix 3.
24	V1833E	V	V	V	5617	T to A	β-Strand 4 of BRCT-C. Residue mutated in germ line (V1833M) and found in two independent clones. Hydrophobic residue conserved in BRCT superfamily.

[a] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[b] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[c] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[d] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[e] According to a BRCA1 BRCT model from Zhang *et al.* (36).

containing plates. Plasmid DNA was isolated from white colonies (which contain only pLex9::BRCA1 COOH-terminal::Tn*4430*), and the insertion of Tn*4430* into the BRCA1 COOH terminal region was confirmed by restriction enzyme mapping. For the identification of Tn*4430* insertions by physical means, pooled plasmid DNA from *E. coli* consisting of the target plasmid into which Tn*4430* was inserted was digested with *Eco*RI and *Bam*HI, enzymes which liberate the BRCA1 insert but do not cut Tn*4430*. This digestion of pooled plasmid DNA generates four fragments: the pLex9 vector backbone, the pLex9 vector containing Tn*4330* insertions, the BRCA1 COOH-terminal fragment, and the BRCA1 COOH terminal fragment containing Tn*4430* insertions. The latter fragment was recovered from an agarose gel and recloned in *Eco*RI-*Bam*HI-digested pLex9 to produce a library of pLex9::BRCA1 COOH terminal domain plasmids containing Tn*4430* insertions in the BRCA1 COOH terminal region. In the case of Tn*4430* insertions identified by either genetic or physical means, following further restriction mapping the bulk of Tn*4430* was deleted from selected clones by digestion with *Kpn*I and religation. The positions of the 15-bp insertions were determined by sequence analysis. Twenty-one plasmids harboring the BRCA1 COOH terminal region with 15-bp insertions were analyzed for transcription activation in *S. cerevisiae* EGY48 containing pSH18–34.

Western Blots. Yeast cells were grown in selective media to saturation, and A_{600} was measured. Cells were harvested and lysed in cracking buffer [8 M urea, 5% SDS, 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, and 0.4 mg/ml bromphenol blue; used 100 μl/7.5 total A_{600}] containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a polyvinylidene difluoride membrane. The blots were blocked overnight with 5% skim milk using TBS-Tween and incubated with the α-pLexA (for LexA constructs) or α-GAL4 DBD (for GAL4 constructs) monoclonal antibodies (Clontech) using 0.5% BSA in TBS-Tween. After four washes, the blot was incubated with the α-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-Tween. The blots were developed using an enhanced chemiluminescent reagent (DuPont NEN, Boston, MA).

RESULTS

Germ-Line Mutations. We analyzed missense mutations occurring in the region from amino acid 1560 to amino acid 1863 described in the Breast Cancer Information Core[5] database. To date, 63 missense variants representing mutations in 55 different residues have been documented, most of which have not been characterized either as disease-associated or as benign polymorphisms. Only four missense mutations have been either confirmed or considered very likely to be associated with disease: A1708E (31–33), P1749R (34), R1751Q (33), and M1775R (4, 31, 35). Three of these four mutations target hydrophobic residues that are conserved in canine, mouse, and rat Brca1. Amino acid composition analysis of this region reveals that only 39% of the residues are hydrophobic. Thus, although the number of characterized mutations is limited, it suggests a preference for loss-of-function mutations to target hydrophobic residues.

Mutagenesis Strategies. To shed light on the critical residues and regions necessary for function, we used four complementary strategies: (*a*) error-prone PCR mutagenesis followed by a screen for loss of function; (*b*) pentapeptide insertion mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus.

Error-prone PCR Mutagenesis Reveals Critical Residues for Activation. Approximately 10^5 yeast clones were screened for loss of transcription activation function. Sixty-two clones were isolated that had lost activity, most of which contained small insertions or deletions causing frameshift mutations and premature termination of the BRCA1 protein, as subsequently confirmed by SDS-PAGE and Western blot analysis (not shown). Two independent clones displayed the same nonsense mutation (Y1769X). Four clones had two mutations (E1660G/M1689K, K1727R/L1786P, S1722P/N1774Y, and S1715N/Q1811L), limiting their further characterization. The 10 remaining clones each had a single missense mutation (one clone also had a silent mutation) and corresponded to eight distinct mutations (Table 1). Interestingly, the screen revealed that hydrophobic residues were the major targets of mutation (six of eight). Furthermore, all of the targeted residues are perfectly conserved in canine, mouse, and rat Brca1 (Table 1). Even conservative mutations may not be well accepted in residues that are perfectly conserved in all species. This is illustrated by mutation F1761I, where a smaller hydrophobic residue is not tolerated in place of a bulkier one. Loss-of-function mutations were located primarily in the BRCT domains. In particular, mutations that occur in BRCT-C [the most COOH-terminal BRCT (amino acids 1756–1855); BRCT-N (amino acids 1649–1736) is located NH_2-terminally to BRCT-C] are in residues that constitute the hydrophobic clusters conserved in the BRCT superfamily. Western blot analysis of the mutant clones (three independent clones of each) revealed that all of the mutants were expressed at levels comparable with the wild type, ruling out the possibility that loss of function was attributable to instability of the protein (Fig. 1). It is important to stress, however, that protein levels are relatively variable in different yeast clones carrying the same constructs and should only be taken as a rough estimate.

Pentapeptide Scanning Mutagenesis Reveals Buried Regions Necessary for Activity. The BRCA1 COOH terminal region was subjected to pentapeptide scanning mutagenesis in which a variable, 5-amino acid cassette was introduced at random. The resulting set of mutated proteins included mutants that displayed complete loss of activity, mutants with reduced activity, and mutants with similar or

[5] Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

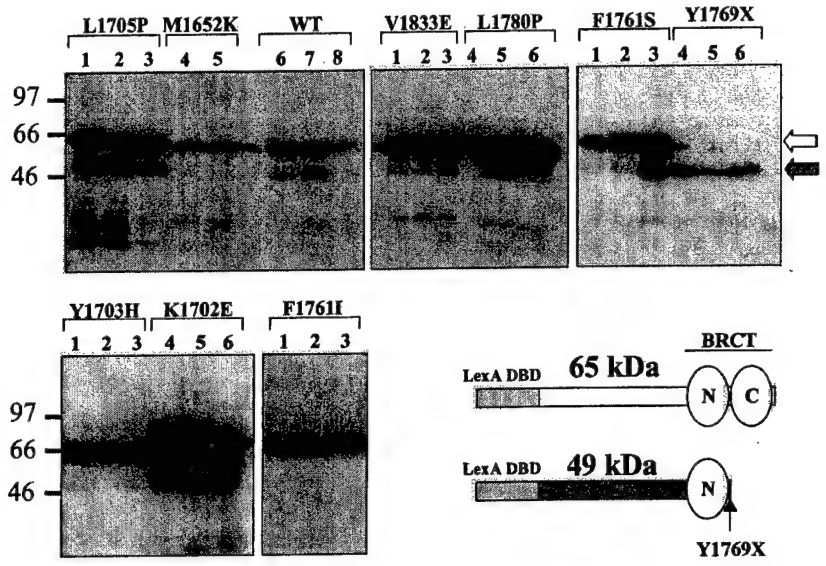

Fig. 1. Expression levels of loss-of-function BRCA1 mutants. Cell lysates containing comparable cell numbers were separated on SDS-PAGE. At least two independent transformants were assayed for each mutant to control for clonal variation. LexA fusion mutant proteins expressed in yeast were detected by Western blot using a monoclonal α-LexA antibody. A schematic representation of the fusion proteins bearing missense mutations [*white arrow*, M_r 65,000 (*65 kDa*)] and a nonsense mutation [*gray arrow*, M_r 49,000 (*49 kDa*)] is shown. Note that mutation Y1769X disrupts the BRCT-C but retains BRCT-N.

higher activity than wild type. Table 2 groups the insertions by location: the first group includes mutations in the region NH_2-terminal to the BRCT domains (amino acids 1560–1649); the second group contains mutations in BRCT-N; and the third group includes mutations in the intervening region between BRCT-N and BRCT-C. The last group includes mutations in BRCT-C. None of the insertions NH_2-terminal to the BRCT domains had a negative effect on transcription activation. Also, insertions in the interval between the BRCT domains or at its boundary (1723RGTPI) had generally less drastic effects. In contrast, all insertions within BRCT-N and several within BRCT-C had a more severe effect. It is clear that BRCT-C tolerates insertions better (only three of five showed loss of activity) than BRCT-N (all mutations reduced activity with six of seven showing drastic impairment). The difficulty in predicting the outcome of mutations can be well exemplified by mutations 1824GGTPI and 1822GVPLH. Both of these mutations target residues at the end of BRCT-C α-helix 2, do not change the net charge of the protein, and are only two residues apart. However, 1822GVPLH has ~6% of the wild-type activity, whereas 1824GGTPI has an activity ~80% higher than wild type. Interestingly, the 1793GVPLK insertion increased transcriptional activation ~4-fold, suggesting that this region of BRCA1 may directly contact a component of the transcription machinery. The pentapeptide mutagenesis results demonstrated that, in addition to substitution mutations, insertion mutagenesis in the COOH-terminal region, particularly in the BRCT domains, can profoundly alter transcriptional activity by BRCA1.

Table 2 *Transcriptional activity of insertion mutants*

Pentapeptide insertion	Miller units[a]	Probable secondary structure element[b]
Empty vector	4.1 ± 3.0	
Wild-type	99.9 ± 14.1	
1571SEGYP	**98.2 ± 98.2**	**Unknown**
1578PSGVP	**120.1 ± 52.6**	**Unknown**
1602PQGVP	**99.3 ± 15.9**	**Unknown**
1620DRGTP	**127.1 ± 11.0**	**Unknown**
1625NGVPH	**81.7 ± 8.2**	**Unknown**
1627MGVPP	**94.4 ± 6.8**	**Unknown**
1665stop	1.6 ± 0.1	α-Helix 1 of BRCT-N
1676RGTPL	2.5 ± 0.2	β-Strand 2 of BRCT-N
1678RGTPN	0.7 ± 0.2	β-Strand 2 boundary of BRCT-N
1695GVPQF	4.3 ± 1.1	β-Strand 3/α-helix 2 loop of BRCT-N
1709GGTPG	1.0 ± 0.7	α-Helix 2/β-strand 4 loop of BRCT-N
1717WGTPF	2.1 ± 0.4	α-Helix 3 of BRCT-N
1723RGTPI	36.5 ± 15.0	α-Helix 3 boundary of BRCT-N
1724GVPLK	10.4 ± 2.5	BRCT-N/BRCT-C interval
1730GVPLN	57.7 ± 7.2	BRCT-N/BRCT-C interval
1737GVPLR	1.0 ± 0.5	BRCT-N/BRCT-C interval
1769GGYPY	11.7 ± 11.1	β-Strand 1/α-helix 1 loop of BRCT-C
1780GVPQL	0.8 ± 0.3	α-Helix 1 of BRCT-C
1793GVPLK	**372.9 ± 113.8**	**β-Strand 2/β-strand 3 turn of BRCT-C**
1822GVPLH	5.7 ± 0.5	α-Helix 2 of BRCT-C
1824GGTPI	**178.0 ± 34.4**	**α-Helix 2 boundary of BRCT-C**

[a] Mutants in bold displayed activity equal to or higher than wild type.
[b] According to a BRCA1 BRCT model from Zhang *et al.* (36).

Characterization of Germ-Line Mutations. To assess the activity of variants that have already been documented but not characterized, we decided to introduce a set of mutations and assay for transcription activation in yeast (Table 3). Mutations T1561I and L1564P are both located in the region preceding the BRCT domains and displayed wild-type activity. L1564P was expected to be a polymorphism because proline is the residue found in the rat Brca1 sequence. The three remaining variants are localized to the BRCT domains. Two variants, D1733G and P1806A, displayed wild-type activity and are suggested to be benign polymorphisms. D1733G introduces a glycine that probably does not affect BRCT structure. P1806A involves a conservative change, and it is important to note that the rat Brca1 sequence has leucine in that position. Only one of the variants tested, G1738E, displayed a loss of function phenotype. Thus, we propose that G1738E is a disease-predisposing variant.

Deletion Mutants of COOH-Terminal Residues Define the Minimal Transactivation Domain (MTD). A construct carrying the germ-line mutation Y1853X does not have detectable transcriptional activity in the context of a GAL4 DBD fusion of the BRCA1 COOH terminus (amino acids 1560–1863; Refs. 13 and 15). A construct containing amino acids 1760–1863 can be considered the MTD, defining I1760 as a 5′ border of this domain (13, 15). Thus, the NH_2-terminal border of the MTD coincides closely with the NH_2-

Table 3 *Transcriptional activity of human BRCA1 unclassified variants (amino acids 1560–1863)*

Exon	Mutation	Activity[a]	Dog[b]	Mouse[c]	Rat[d]	Nucleotide[e]	Base change	Probable secondary structure element[f]	Reference
16	T1561I	+	A	T	T	4801	C to T	Unknown	Durocher *et al.* (41)
16	L1564P	+	L	L	P	4810	T to C	Unknown	BIC[g]
20	D1733G	+	D	E	E	5317	A to G	BRCT-N/BRCT-C interval	BIC
20	G1738E	−	G	G	G	5332	G to A	BRCT-N/BRCT-C interval	BIC
23	P1806A	+	P	P	L	5535	C to G	β-Strand 2/β-strand 3 loop of BRCT-C	BIC

[a] At least 10 independent clones were assayed and scored 8 h after the addition of X-gal. +, blue with same intensity as wild-type control. −, white, similar to two (F1761S and Y1769X) loss-of-function controls.
[b] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[c] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[d] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[e] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[f] According to a BRCA1 BRCT model from Zhang *et al.* (36).
[g] Breast Cancer Information Core.

terminal border of BRCT-C (I1760 is the first conserved hydrophobic residue in the BRCT superfamily). To identify the COOH-terminal border of the MTD, several deletion mutants were made in the amino acids 1560–1863 context and assayed for their ability to activate transcription in yeast. Fig. 2 shows the several deletion mutants analyzed aligned to mouse, rat, dog, and human BRCA1 wild-type sequences. Mutant H1860X introduces a stop codon but maintains all of the conserved amino acids in canine and human BRCA1. P1856X maintains the hydrophobic residues, which are conserved in all of the BRCT domains described in several species. I1855X and L1854X delete one and two conserved hydrophobic residues, respectively. Y1853X is a mutation found in the germ-line of breast and ovarian cancer patients in high-risk families (21). These constructs were transformed into SFY526 and HF7c and analyzed for their ability to activate different reporters (Fig. 2*b*). Activity comparable with the wild-type was obtained with mutants H1860X and P1856X. However, mutations that disrupted the conserved hydrophobic residues (I1855X and L1854X) at the end of the BRCT domain abolished activity. Therefore, we define the MTD in BRCA1 as amino acids 1760–1855. To determine whether the loss of activity by the mutants correlated with the stability of the protein, yeast cells were transformed with the same mutated alleles in a vector conferring high expression (pAS2-1). Transcriptional activity using these constructs (in pAS2-1 backbone) was measured, and results were similar with I1855X showing some residual activity. Expression was highly variable, and mutants were in general expressed at lower levels than wild type (Fig. 2*c*). There was no correlation between loss of activity and lower levels of expression because the transcriptionally active mutant H1860X was expressed at levels lower or comparable with transcriptionally inactive mutants I1855X and Y1853X (Fig. 2*c*).

DISCUSSION

In this report, we describe an extensive mutagenesis analysis of the BRCA1 COOH terminal region and partly define the critical requirements for transcriptional activity by BRCA1. Four complementary strategies were used: (*a*) error-prone PCR mutagenesis, followed by a screen for loss of function; (*b*) pentapeptide scanning mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus. Our results support the notion that there are no particular hot spots for loss-of-function mutations, but rather that these mutations are scattered throughout the coding sequence. Nevertheless, we were able to identify preferential sites critical for activation. An overview of the mutations and their effects is presented in Fig. 3. We discuss the general conclusion of each strategy and then we analyze the possible structural outcome of the mutations based on the crystal structure of XRCC1 BRCT (36).

Error-prone PCR Mutagenesis. Eight distinct BRCA1 mutations were recovered that resulted in loss of transcription activation function. In the course of the screening procedure, many additional clones that displayed a light blue color were noted and were probably mutants with reduced function, but only clones with complete loss of function were analyzed further. No PCR-generated mutations were found in the region external to the BRCT domains, although this constitutes approximately one-third of the tested sequence, indicating a preference for mutations that affect transcription activation to occur in the BRCT domains (Fig. 3).

Six of eight unique PCR-generated mutations were in hydrophobic residues conserved in human, canine, mouse, and rat Brca1 (6, 7), supporting the notion that hydrophobic residues are important for the stability of the BRCT domains and BRCA1 function *in vivo*.

a

BRCA1 C-terminus

BRCT 3' Border

```
..TSVYRCRDLDAYLVQNITCGRDSSEPQDSND   Rat
..SGSYRCRDLDAYLVQNITC..DSSEPQDSND   Mouse
..VALYQCQELDTYLIPQIPRTAADSSQPCV     Dog
..VALYQCQELDTYLIPQIPHSHY            Human
```

b

	Constructs	HF7c (Liquid)	SFY526 (β-gal)
..VALYQCQELDTYLIPQIPHSHY	Wild-type	+ (1.0)	+
..VALYQCQELDTYLIPQIP*	H1860X	+ (1.0)	+
..VALYQCQELDTYLI*	P1856X	+ (1.0)	+
..VALYQCQELDTYL*	I1855X	- (0.0)	-
..VALYQCQELDTY*	L1854X	- (0.0)	-
..VALYQCQELDT*	Y1853X	- (0.0)	-

c

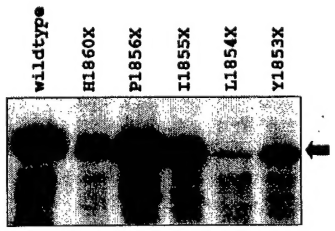

Fig. 2. Deletion analysis of the COOH-terminal region. *a*, alignment of the wild-type sequences of the COOH terminus of rat, mouse, dog, and human BRCA1. Amino acids in *bold* represent conserved residues. *Shaded area*, residues at the 3′ border of the BRCT-C domain. *b*, transcriptional activity of GAL4 DBD fusion deletion constructs, made in the context of BRCA1 amino acids 1560–1863. *S. cerevisiae* (HF7c) carrying the indicated fusion proteins were assayed for growth in the absence of tryptophan and histidine in liquid medium. Activity relative to cells growing in medium lacking tryptophan alone after 36 h is shown in *parentheses*. Filter β-galactosidase assays for SFY526 were scored at 12 h after X-gal addition. At least four independent clones were assayed for each construct. *c*, Western blot showing levels of protein expression of the different constructs (*black arrow*) detected by a α-GAL4-DBD monoclonal antibody.

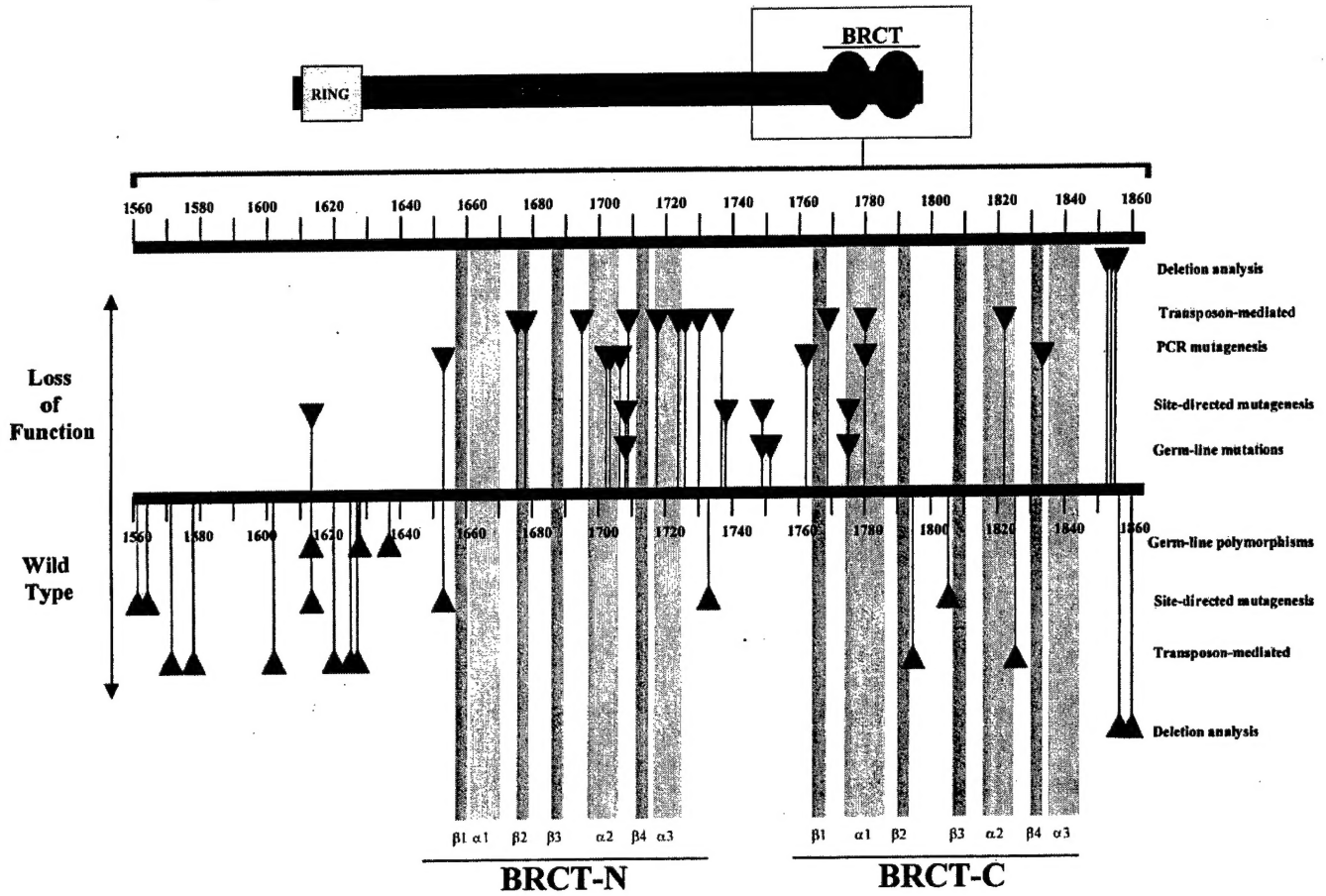

Fig. 3. Domain structure of the BRCA1 COOH terminal region (amino acids 1560–1863) and characterized mutations. *Top panel,* a schematic representation of full-length BRCA1 protein featuring the RING domain (*yellow box*) in the NH_2-terminal region and the BRCT domains (*red circles*) in the COOH-terminal region. The region analyzed in this study is contained in the *red box,* which is enlarged and represented in the *bottom panel. Purple* and *pink bars,* predicted β-strands and α-helices, respectively. Secondary structure predictions were made by Zhang *et al.* (36) based on the crystal structure of the XRCC1 BRCT domain. Mutations represented in the *upper part* (*red triangles*) result in loss of function, whereas mutations in the *lower part* (*green triangles*) result in activity equal or higher than wild type. Germ-line mutations and polymorphisms are variants defined by genetic linkage to be disease-associated and benign polymorphisms, respectively. Site-directed mutagenesis, PCR mutagenesis, transposon-mediated mutagenesis, and deletion analysis represent mutations that have been characterized by transcription activation assay in yeast to be either loss of function (*upper part*) or wild type (*lower part*).

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a method by which a variable 5-amino acid cassette is introduced at random into a target protein (29, 30, 37). This approach differs from error-prone mutagenesis because clones are not selected for loss of activity but rather mutations are analyzed only after they have been generated. Therefore, mutants with gain of function, loss of function, and novel activities can be produced (30, 37). Moreover, it has been shown that insertion is essentially random (29). The results obtained are in agreement with the PCR-mediated mutagenesis in that the region NH_2-terminal to the BRCT domains (amino acids 1560–1649) seems to be more tolerant of mutation; none of six different pentapeptide insertions in this region affected transcription activation. The fact that derivatives containing insertion mutations in this region retained wild-type activity suggests that this region is nonglobular and is probably a flexible part of the COOH-terminal region without many critical secondary structure elements. In fact, the region encompassing amino acids 1524–1661 is predicted to be nonglobular (5). The pentapeptide mutagenesis results also suggest that changing the net charge of the protein does not necessarily correlate with an alteration in transcription activity, as would be expected for classical acidic activators (38), because 1793GVPLK (which adds a positive charge) shows a 4-fold increase in activity. Interestingly, only 4 of the 63 COOH-terminal germ-line variants involve nonconservative substitutions in acidic residues, thought to be important for activation, suggesting that, contrary to initial predictions, BRCA1 may not be a classical acidic activator (4). The 1793GVPLK mutation, which is hyperactive for transcription activation, may define a point of contact between the BRCA1 COOH-terminal region and the transcription machinery.

Deletion Analysis. Our analysis demonstrates that residues COOH-terminal to amino acids 1855 are dispensable for activation, consistent with the extreme evolutionary divergence of those residues (Fig. 2; Refs. 39 and 40). The results also underscore the importance of the last hydrophobic cluster in the sequence (YLI for human and canine; YLV for mouse and rat) and provide a plausible explanation for the complete loss of function (*in vitro* and *in vivo*) of Y1853X alleles.

Site-directed Mutagenesis. Only one of five germ-line mutations analyzed displayed loss of function, suggesting that a large part of variants in the COOH-terminal region will probably be benign polymorphisms, including some variants found in the BRCT domains. Very little data are available at this moment to confirm or contradict the results obtained. In particular, T1561I illustrates the difficulties involved in predicting outcome from population data. T1561I was found in one affected individual but not in control individuals (41). This could suggest that T1561I is a disease-predisposing variant. However, although found as a germ-line mutation, it was absent from

the tumor from the same patient (41), indicating that this mutation is a benign polymorphism.

Structural Basis for Effects of BRCT Domain Mutations. The COOH-terminal BRCT domain of XRCC1 consists of a four-stranded parallel β-sheet (β1–β4) surrounded by three α-helices (α1–α3; Ref. 36). The β-sheet forms the core of the structure with a pair of α-helices (α1 and α3) on one side of the β-sheet and the remaining α-helix (α2) on the other side. A model of the more COOH-terminal BRCT domain of BRCA1 has been constructed based on the crystal structure of the BRCT domain of XRCC1 (36). This model allows an interpretation of the effect of some of the mutations described in this study (Tables 1–3) on BRCT domain structure (Fig. 3).

The position of the M1652K mutation corresponds to a position (Asp4) in the XRCC1 structure that is thought to form a salt bridge at the BRCT dimer interface (36). Although M1652 would not be expected to be involved in salt bridge formation at neutral pH, residues in this region nevertheless may also be involved in homo- or heterodimer formation in BRCA1.

Missense mutations at positions 1702, 1703, and 1705 of the BRCT-N domain and a pentapeptide insertion at position 1822 of the BRCT-C domain abolish transcription activation by the BRCA1 COOH terminus (Tables 1 and 2). These mutations are predicted to occur in a region of highly variable length and composition that encompasses helix α2 in BRCT domains (36). It was suggested that this variability indicated that this region was not involved in formation of the core fold of the BRCT domain (36). Nevertheless, the mutations isolated here reveal that this region of the BRCT domain is critical for the transcription activation function of the BRCA1 COOH terminus.

Residue F6 forms part of a highly conserved hydrophobic pocket centered on residue W74 in helix α3 in the COOH-terminal BRCT domain of XRCC1 (36). Mutations at the corresponding position (F1761) in the BRCT-C domain of BRCA1 abolish transcription activation (Table 1). By analogy with XRCC1, residue F1761 of BRCA1 is also predicted to form part of a hydrophobic pocket, the disruption of which by mutation may compromise correct BRCT domain folding. In contrast, residue L25 is implicated in the interactions between helices α1 and α3, which form a paired helical bundle in the three-dimensional structure of the BRCT domain of XRCC1 (36). A missense mutation of the corresponding residue (L1780) or a pentapeptide insertion at this position in the BRCT-C domain of BRCA1 abolishes transcription activation by the BRCA1 COOH terminus region (Tables 1 and 2). These mutations are likely to affect the interactions between helices α1 and α3, thereby destabilizing the BRCT domain structure. Two other missense mutations in the BRCT-C domain, P1806A and V1833E, were shown, respectively, to display wild-type activity and to abolish transcription activation (Tables 1 and 3). Interestingly, P1806A is predicted to have no obvious effect on the structure, whereas a less drastic mutation at position V1833 (to methionine) has been predicted to destabilize the fold of the domain (36), suggesting that V1833E will behave similarly.

Pentapeptide insertions in many of the predicted secondary structure elements in the COOH-terminal region of BRCA1 abolish transcription activation (Table 1 and Fig. 3). Some of these insertions are likely to disrupt formation of the correct BRCT domain core fold, *e.g.*, insertions in strand β2 (1676RGTPL) and in helices α2 (1822GVPLH) and α3 (1717WGTPF). In contrast, the 1780GVPQL insertion in helix α1 is predicted to be at the BRCT dimer interface and thereby may affect the association of this domain with another protein, *e.g.*, RNA helicase A, which interacts with BRCA1 through residues in helix α1 (17).

Different Roles of BRCT-N and BRCT-C. Our insertion mutagenesis results suggest that BRCT-C can tolerate insertions better than BRCT-N without affecting transcription activation function. In addition, BRCT-N is more highly conserved in other species than is BRCT-C (39, 40), suggesting a higher constraint for function. The BRCT-N seems to be very important for binding to RNA helicase A (17), although it seems to lack an independent activation domain (mutant Y1769X is inactive). The borders of BRCT-C coincide well with the limits of the MTD, but only in combination with BRCT-N are high levels of activation achieved (13). It is tempting to speculate that BRCT-N is involved in the interaction of BRCA1 with RNA helicase A and is responsible for presenting BRCT-C in a correct way to obtain a transcriptionally competent activator.

Functional Assay. We have performed an extensive analysis of the BRCA1 COOH terminal region (amino acids 1560–1863) and have found that there is a correlation between loss of transcription activation function and the human genetic data, suggesting that the assay could be used to predict the effect of missense mutations in this region. Although the effects of mutations on transcriptional activity have been found to be comparable in yeast and mammalian cells (13, 15), it is possible that the effect of some mutations may be evident only in mammalian cells, *e.g.*, because of an interaction with mammalian-specific regulators, raising the possibility of a misinterpretation of the data obtained in yeast.

In the results presented here for substitution mutations, we have used a reporter gene with relatively low stringency (eight Lex operators; Ref. 26). The rationale for this choice was to recover only mutants that cause dramatic reduction or complete loss of activity. Mutations that partially disrupt the function would still activate the reporter. In the absence of knowledge of the minimum *in vivo* threshold of transcription activity needed for tumor suppression, it would be inappropriate to make decisions on whether a particular mutation would represent a wild-type or a cancer predisposing allele. For example, a particular mutation that shows 50% loss of activity in yeast could still be perfectly functional in breast and ovarian cells.

In conclusion, the data presented here suggest that the yeast assay for monitoring transcription activation by BRCA1 will provide a wealth of functional information in a research setting. That includes identifying protein-protein interaction regions, defining critical residues for activity, and providing tools to identify possible regulators. A general use of the assay to help in risk assessment and providing information for clinical decisions must await further confirmation from population-based studies.

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